



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Markou et al.

Application No.: 10/527,525

Filed: Oct. 14, 2005

For: Methods for treating disorders
associated with mGlu receptors
including addiction and depression

Art Unit: 1617

Examiner: K. Carter

DECLARATION OF DR. ATHINA MARKOU
UNDER 37 CFR § 1.131

Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Athina Markou, states as follows:

I. I am one of the co-inventors of the above-captioned patent application. I am a Professor at the Department of Psychiatry, at the University of California, San Diego and Adjunct Professor at the Molecular and Integrative Neurosciences Department of The Scripps Research Institute. I have published more than 120 journal articles and book chapters, and have received the Efron Award from the American College of Neuropsychopharmacology. I am Section Editor of *Neuropharmacology*, and on the editorial boards of eight other journals, including *Biological Psychiatry*, *Neuropsychopharmacology*, *Behavioral Neuroscience* and *The American Journal on Addictions*. I am also Director of a NIH consortium on the discovery of treatments for depression and nicotine dependence, as well as Principal Investigator on several research grants on nicotine dependence funded by the National Institute on Drug Abuse and the State of California. My curriculum vitae is attached as Exhibit A.

2. I understand that the Examiner has rejected the claims pending in the subject patent application as being obvious. I understand that the Examiner's primary belief underlying the rejection is that both mGluR2/3R antagonists and mGluR5 antagonists have been used to treat drug addiction and substance abuse, and, therefore, it would be obvious to combine a mGluR2/3R antagonist and a mGluR5 antagonist to treat addictive disorders as presently claimed.

3. I understand that the Examiner has cited Fundytus et al., "Attenuation of morphine withdrawal symptoms by subtype-selective metabotropic glutamate receptor antagonists," *British J. Pharmacol.* 120:1015-20, 1997, and believes that Fundytus et al. reports treatment of withdrawal symptoms with a mGluR2/3 and mGluR5 dual antagonist MCPG.

4. It is my professional opinion that, prior to the subject invention, it would not be obvious to combine a mGluR2/3R antagonist and a mGluR5 antagonist to treat addictive disorders. My opinion is based on the following scientific knowledge and literature reports.

(i) mGlu5 receptors are located postsynaptically. On the other hand, mGlu2/3 receptors are located presynaptically. This is illustrated in the publication by Schoepp, "Unveiling the functions of presynaptic metabotropic glutamate receptors in the central nervous system," *J. Pharmacol. Exp. Ther.* 299:12-20, 2001 (attached as Exhibit B). The localization of mGluR2/3 and mGluR5 receptors indicates that antagonist actions at the mGluR2/3 increase glutamate transmission, while antagonist actions at the postsynaptic mGluR5 decrease glutamate transmission.

(ii) There are a number of references from the literature suggesting that one should expect opposite neurochemical and behavioral effects of metabotropic glutamate 2/3 receptor (GluR2/3) antagonists and metabotropic glutamate receptor 5 (mGluR5) antagonists. Neurochemically, Mills et al. (*J. Neurochem.* 79: 835-48, 2001; attached as Exhibit C) examined the role of mGluRs in the increase in extracellular excitatory amino

acids following spinal cord injury, and found that the mGluR5 antagonist MPEP decreased excitatory amino acid concentrations, while treatment with the mGluR2/3 agonist LY 341495 increased excitatory amino acid levels. Xi et al. (J. Pharmacol. Exp. Ther. 300:162-71, 2002; attached as Exhibit D) reported that the mGluR2/3 antagonist LY143495 increased extracellular glutamate in the nucleus accumbens. By contrast, the mGluR5 antagonist MPEP inhibited glutamate release in vitro and in vivo in the corpus striatum (Thomas et al., Neuropharmacology 41: 523-7 2001; attached as Exhibit E) and the periaqueductal grey (de Novellis et al., Eur J Pharmacol 462: 73-81 2003; attached as Exhibit F). Behaviorally, Sharko et al. (Alcohol. Clin. Exp. Res. 32: 67-76, 2008; attached as Exhibit G) studied whether mGluRs modulate the acute sedative-hypnotic properties of ethanol in C57BL/6J mice. The authors found that the mGluR5 antagonist MPEP significantly enhanced both the sedative and hypnotic effects of ethanol, while the mGluR2/3 antagonist LY341495 significantly decreased the sedative hypnotic effects of ethanol.

5. Substance abuse and substance dependence are related but different concepts. Continued substance abuse can often lead to development of substance dependence (or addiction). When subjects with substance dependence cease substance use (i.e., withdrawal), they will usually develop withdrawal symptoms (e.g., depression). It is important to note that substance abuse and substance dependence may usually require different means for treatment and intervention.

6. Fundytus et al. cited by the Examiner does not report that MCPG is effective in treating withdrawal symptoms in rats with morphine dependence. Instead, Fundytus et al. disclosed data which indicate that MCPG prevented the development of morphine dependence in rats which were simultaneously administered with morphine (see, e.g., Fig. 1 of Fundytus et al.).

7. Fundytus et al. reported that treatment with mGluR antagonists, including MCPG, has no effect on withdrawal symptoms in rats that have already developed morphine dependence. This is stated in Fundytus et al., e.g., at page 1018, left column, second and third paragraphs.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: Sept 2, 2008 Athina Markou
Athina Markou, Ph.D.

Attachments: Exhibits A-G.

**CURRICULUM VITAE****ATHINA MARKOU, Ph.D.****WORK ADDRESS**

Department of Psychiatry, 0603
University of California, San Diego
9500 Gilman Drive
La Jolla, California 92093, U.S.A.

EDUCATION

- 1979-1983 B.A. with High Distinction (Psychology), Deree College, The American College of Greece, Aghia Paraskevi, Athens, Greece
- 1983-1986 M.A. (Physiological Psychology), Department of Psychology, University of Cincinnati, Cincinnati, Ohio, U.S.A.
- 1986-1991 Ph.D. (Physiological Psychology), Department of Psychology, University of California, San Diego, La Jolla, California, U.S.A.

PROFESSIONAL EXPERIENCE

- 1991-1994 **Research Associate** (Postdoctoral Fellow), Department of Neuropharmacology, The Scripps Research Institute, La Jolla, California, U.S.A.
- 1991, 1994, 1995 **Visiting Scientist**, Departments of Anatomy and Experimental Psychology, University of Cambridge, Cambridge, England, United Kingdom.
- 1994-1999 **Assistant Professor**, Department of Neuropharmacology, The Scripps Research Institute, La Jolla, California, U.S.A.
- 1997-2001 **Assistant Adjunct Professor** (non-salaried), Department of Psychiatry, School of Medicine, University of California, San Diego La Jolla, California, U.S.A.
- 1998-Present **Member**, Ph.D. Group in Neurosciences, School of Medicine, University of California, San Diego, La Jolla, California, U.S.A. (non-salaried)
- 1999-2006 **Associate Professor**, Department of Neuropharmacology, The Scripps Research Institute, La Jolla, California, U.S.A.
- 2001-2006 **Associate Adjunct Professor** (non-salaried), Department of Psychiatry, School of Medicine, University of California, San Diego, La Jolla, California, U.S.A.
- 2002-2008 **Field Editor** (member of Executive Board; non-salaried), *Neuropharmacology*.

2003-2004	Guest Editor (non-salaried), “Animal Models of Depression and Antidepressant Activity” (special issue, published 2005), <i>Neuroscience and Biobehavioral Reviews</i> .
2003–Present	Director , National Cooperative Drug Discovery Group for the Treatment of Mood Disorders and Nicotine Addiction, National Institutes of Health (consortium grant).
2004-2008	Panel Member , Neurobiology of Motivated Behavior Study Section, National Institutes of Health.
2005	Guest Editor (non-salaried), “Metabotropic Glutamate Receptors” (special issue), <i>Neuropharmacology</i>
2006-Present	Adjunct Professor , Molecular and Integrative Neurosciences Department, The Scripps Research Institute, La Jolla, California, U.S.A.
2006-Present	Professor , Department of Psychiatry, School of Medicine, University of California, San Diego, La Jolla, California, U.S.A.
2008-Present	Section Editor , <i>Neuropharmacology</i>

GRANTS AND FELLOWSHIPS (active grants in bold)

Graduate Summer Research Fellowship, Graduate Office, University of Cincinnati, U.S.A. (1985)

Parkinson's Disease Foundation Summer Research Fellowship, U.S.A. (1988)

Individual National Research Service Award Predoctoral Fellowship, National Institute on Drug Abuse, U.S.A. (1988 - 1991) - direct costs: \$11,500/year for 3 years. Title: Cocaine withdrawal in the rat: Measures of brain reward. Principal Investigator: Athina Markou.

Individual National Research Service Award Postdoctoral Fellowship, National Institute on Drug Abuse, U.S.A. (1991 - 1994) - approximate direct costs: \$26,000/year for 3 years. Title: Neurochemical bases of reward and reward representations. Principal Investigator: Athina Markou.

Research Scientist Career Development Award (K21), National Institute on Drug Abuse, U.S.A. (1994 - 1999) - direct costs: \$95,000/year for 5 years. Title: The role of the amygdala in reward processes. Principal Investigator: Athina Markou.

R01 Research Grant, National Institute on Drug Abuse, U.S.A. (1995 - 1999) - approximate direct costs: \$155,000/year for 4 years. Title: Neuronal substrates of cocaine reward. Principal Investigator: George F. Koob, Co-Investigator: Athina Markou

Research Grant 1106 from Sandoz/Novartis Pharma Inc., Basel, Switzerland (1995 – 2004; renewed competitively twice with no gap in funding) - approximate direct costs: \$140,000/year for the first 3 years (1995 –1998) and approximate direct costs: \$400,000/year for the next 5 years (1998 – 2004). Title of the first two grants: Neurobiology of reward and motivational processes: Depressive symptomatology as seen across diagnostic categories with relevance to depression and schizophrenia. Title of the third grant: The role of metabotropic glutamate receptors in depression, nicotine addiction and cognition. Principal Investigator: Athina Markou.

Research Grants 7RT-0004, 10RT-0074, 12RT-0231, 15RT-0022 from the State of California Tobacco-Related Disease Research Program, U.S.A. (1998 – 2009; renewed competitively three times with no gap in funding) - approximate direct costs: \$150,000/year for 11 years. Title: Neurobiological substrates of nicotine addiction. Principal Investigator: Athina Markou.

- 2 R01 DA11946 Research Grant from the National Institute on Drug Abuse (2000 - 2010) - direct costs: \$125,000/year for 10 years. Title: Neurobiology of nicotine reward and withdrawal. Principal Investigator: Athina Markou. Renewed once competitively with no gap in funding.**
- 1 R01 MH62527 Research Grant from the National Institute of Mental Health (2002 - 2008) - direct costs: \$175,000/year for 5 years. Title: Negative symptoms of schizophrenia: animal models. Principal Investigator: Athina Markou.**
- U01 MH69062 National Cooperative Drug Discovery Group for the Treatment of Mood Disorders or Nicotine Addiction (NCDDG-MD/NA) consortium grant jointly funded by the National Institute of Mental Health and the National Institute on Drug Abuse (2003-2008; one year no-cost extension). Title: Development of GABA_B compounds for depression and smoking. Direct costs for Markou component: \$250,000 – 330,000/year for 5 years. Total budget for 5 years (includes direct and indirect costs): 3.5 million. Director of the overall project and Principal Investigator of one component: Athina Markou. [SELECTED BY THE SCRIPPS RESEARCH INSTITUTE FOR PRESS RELEASE].**
- 14IT-0053 Innovative Developmental and Exploratory Award (IDEA) grant from the Tobacco-Related Disease Research Program of the State of California, U.S.A. (2005-2007) - direct costs: \$ 50,000/year for two years. Title: Development of nicotine withdrawal measures in mice. Principal Investigator: Svetlana Semenova (Staff Scientist in Markou laboratory). Co-investigator: Athina Markou.**
- 1 R01 MH073923 Research Grant from the National Institute of Mental Health (2006-2007) – direct costs: \$170,000/year for four years. Title: The 5-HT₇ receptor in models of psychiatric disorders. Principal Investigator: Peter Hedlund (Assistant Professor in Department of Molecular Biology, The Scripps Research Institute). Co-investigator: Athina Markou**
- 1 R21 MH080001 Research Grant from the National Institute of Mental Health (2007 – 2009) – direct costs: approximately \$137,000/year. Title: Simulated human pharmacokinetics in rat: methylphenidate. Principal Investigator: Ronald Kuczenski (Professor, Department of Psychiatry, UCSD). Co-investigator: Athina Markou**
- 1 RO1 DA023209 Research Grant from the National Institute on Drug Abuse (2007 – 2012) – direct costs: approximately \$175,000/year. Title: Neurobiology of nicotine reward and dependence in mice. Principal Investigator: Athina Markou.**
- 1 RO1 DA023926 Research Grant from the National Institute on Drug Abuse (2007 – 2010) – direct costs: approximately \$250,000/year. Title: Modulators of metabotropic glutamate receptor subtype 2 for cocaine dependence. Principal Investigator: Nicholas Cosford (Director of Medicinal Chemistry Core Facility, Burnham Institute, La Jolla, California, USA). Principal Investigator of Subcontract to UCSD: Athina Markou.**
- R01 DA001568-29 Research Grant from the National Institute on Drug Abuse (2008 – 2013) – direct costs: approximately \$ 250,000/year. Title: Behavioral characterization of acute and chronic amphetamine. Principal Investigator: Ronald Kuczenski (Professor, Department of Psychiatry, UCSD). Co-investigator: Athina Markou**
- R21 MH078979 Research Grant from the National Institute of Mental Health (2008 – 2010) – direct costs: approximately \$150,000/year. Title: Translational measures of anhedonia in humans and rats. Co-Principal Investigator: Athina Marou (in collaboration with Dr. Diego Pizzagalli, Department of Psychology, Harvard University).**

AWARDS AND HONORS

Travel Award from the European Behavioural Pharmacology Society (EBPS) to attend the 2nd meeting of the Society (1988)

Award in Poster Presentation from the European Behavioural Pharmacology Society (EPBS) at the 3rd meeting of the Society (1990)

National Institute on Drug Abuse Travel Award to attend the 53rd Annual Conference of the College on Problems of Drug Dependence (CPDD) (1991)

National Institute on Drug Abuse Travel Award to attend the 55th Annual Conference of the College on Problems of Drug Dependence (CPDD) (1993)

American College of Neuropsychopharmacology / Mead Johnson Travel Award to attend the 34th Annual Meeting of the American College of Neuropsychopharmacology (ACNP) (1995)

Travel Award from the International Program of the National Institute on Drug Abuse to participate at the International Conference on Neuroscience and Addictions organized by the Ministry of Health of Mexico in consultation with the World Health Organization, Mexico City, Mexico (2002).

Japanese Program for the Invitation of Foreign Scientists to Japanese Institutes by the Society of Japanese Pharmacopeia to give a series of seminars at the Japanese Neuroscience meeting (Nagoya), International Society for Neurochemistry/Asian Pacific Society for Neurochemistry (Kyoto), Kyoto University, and the Brain RIKEN Institute, Tokyo (2003).

Daniel H. Efron Research Award: Honorific Award given by the American College of Neuropsychopharmacology (ACNP) yearly to a scientist 45 years of age or younger for outstanding basic/translational research contributions to neuropsychopharmacology. Specifically, “An exceptionally bright, talented, and creative researcher, Dr. Markou has made significant contributions to the interface between the neuropsychopharmacology of reward in animal models and human psychopathology” (2004).

Selected by Deree College – The American College, where I completed my B.A. degree, to be featured as a “success story” in ads in several national high circulation weekend editions of greek newspapers (Summer 2007).

Kenny, P.J., Chartoff, E., Roberto, M., Carlezon Jr., W.A. and Markou, A. (2008) NMDA receptors regulate nicotine-enhanced brain reward function and intravenous nicotine self-administration: Role of the ventral tegmental area and central nucleus of the amygdale. *Neuropsychopharmacology*. In press. [ARTICLE FEATURED AT FACULTY OF 1000 BIOLOGY].

Selected as the honorary speaker at the annual meeting of the Greek Neuroscience Society that takes place yearly in association with the annual Society for Neuroscience meeting (October 2008).

Received Certification of Appreciation by the journal *Biological Psychiatry* for “dedicated service and valuable contributions as one of the Top 10 Reviewers for *Biological Psychiatry* in 2007”.

Received Service Award by the journal *Neuropsychopharmacology* “in recognition of valuable contributions” to the journal for 2006 (continue to be among the top reviewers for this journal).

ADVISORY ACTIVITIES

Journal Editor and Membership in Editorial boards

Editorships

2002 – 2008	Editor (member of the Executive Board), <i>Neuropharmacology</i>
2003-2004	Guest Editor , “Animal Models of Depression and Antidepressant Activity” (special issue, published 2005), <i>Neuroscience and Biobehavioral Reviews</i> .
2005	Guest Editor , “5th International Meeting on Metabotropic Glutamate Receptors” (special supplement issue; published 2005), <i>Neuropharmacology</i> (papers and abstracts)
2007 – Present	Reviewing Editor , <i>Frontiers in Behavioral Neuroscience</i>
2008 – Present	Section Editor , <i>Neuropharmacology</i>

Editorial Board Memberships

2003 - Present	Editorial Board Member , <i>The American Journal on Addictions</i>
2005 – Present	Editorial Board Member , <i>Biological Psychiatry</i>
2005 – Present	Faculty Member , <i>Faculty of 1000 Medicine</i>
2007 – Present	Editorial Board Member , <i>Neuroscience and Biobehavioral Reviews</i>
2008 – Present	Editorial Board Member , <i>Open Journal of Neuropsychopharmacology</i>
2008 – Present	Editorial Board Member , <i>Neuropsychopharmacology</i>
2008 – Present	Consulting Editor , <i>Behavioral Neuroscience</i>

Journal Reviewer for:

Alcohol; Behavioral Neuroscience; Behavioural Brain Research; Behavioural Pharmacology; Biological Psychiatry; Brain Research; Brain Research Bulletin; Brain Research Interactive; British Journal of Pharmacology; Current Psychiatry Reviews; Drug and Alcohol Dependence; European Journal of Neuroscience; European Journal of Neuropsychopharmacology; Experimental and Clinical Psychopharmacology; Journal of Neuroscience; Journal of Psychiatric Research; Life Sciences; Molecular Psychiatry; Nature; Nature Reviews Neuroscience; Neurobiology of Disease; Neuropharmacology; Neuropsychopharmacology; Neuroscience; Neuroscience and Biobehavioral Reviews; Neuroscience Letters; Neurotoxicity Research; Nicotine and Tobacco Research; Pharmacology, Biochemistry and Behavior; Physiology and Behavior, Proceedings of the National Academy of Sciences USA; Psychopharmacology; Synapse; The American Journal on Addictions; Trends in Pharmacological Sciences.

Grant Reviewer:

National Institute on Drug Abuse (NIDA) RFA special grant review panel on Drug Craving (December 1996)

Ontario Mental Health Foundation of Canada (December 1998; December 1999; December 2001; December 2002)

National Institute on Drug Abuse (NIDA) RFA special grant review panel on Genetics of Drug Addiction Vulnerability (June 1999)

National Cancer Institute (NCI) and National Institute on Drug Abuse (NIDA) RFA special grant review panel on Transdisciplinary Tobacco Use Research Centers (July 1999)

Swiss Federal Institute of Technology (June 2000)

Veterans Administration, External Merit Review (August 2000)

National Science Foundation (October 2000; October 2001)

National Institute on Drug Abuse (NIDA) RFA special review panel on The Development of Behavioral Methods for Drug Abuse Studies in the Mouse (November 2000)

National Institute on Drug Abuse (NIDA) review of Cutting-Edge Basic Research Awards (CEBRA) application (May 2001; January 2005)

Arizona Disease Control Research Commission (2001 – Present; annually)

The Wellcome Trust, London, England, United Kingdom (January 2003)

Ad hoc member of the National Institute on Drug Abuse (NIDA) Medication Development Research Subcommittee (February 2003, June 2004)

National Institute on Drug Abuse (NIDA) reviewer for Behavioral Science Track Award for Rapid Transition (B-START) program grant applications (March 2003, October 2003, August 2005, November 2006; December 2007)

National Institute on Drug Abuse (NIDA) site visit/review for Center grant application (May 2003)

Science Foundation Ireland (February 2004)

National Institutes of Health (NIH) member of the Neurobiology of Motivated Behaviors (previously Functional and Integrative Cognitive Neuroscience 1) Study Section. Washington DC (February 2004 ad hoc member; June 2004 ad hoc member; 2004 - 2008 enpaneled member).

Medical Research Council (MRC), United Kingdom (April 2004)

National Institute on Drug Abuse (NIDA) special study section for P01 Program Project grant application (November 2004; November 2005)

National Institute of Mental Health (NIMH) special study section for Conti Schizophrenia Centers (March 2005)

Center for Scientific Review Special Emphasis Panel, National Institutes of Health (November 2005)

Center for Scientific Review Special Emphasis Panel on Interdisciplinary Developmental Science Centers for Mental Health, National Institutes of Health (June 2006)

Center for Scientific Review Special Emphasis Panel on National Cooperative Drug Discovery Groups for the Treatment of Mental Disorders, Drug or Alcohol Addiction (NCGGG), National Institutes of Health (November 2007)

Committee and Task Force Memberships:

2000 - 2003	Credentials Committee, College on Problems of Drug Dependence (CPDD)
2001 - 2003	Program Committee, American College of Neuropsychopharmacology (ACNP)
2002 - 2005	Program Committee, International Behavioral Neuroscience Society (IBNS)
2003 - 2005	Program Committee, Society for Research on Nicotine and Tobacco (SRNT) Reviewer for SRNT Program Committee (2007)
2003 - 2006	Program Committee, College on Problems of Drug Dependence (CPDD)
2004	Ad hoc Committee, Society for Research on Nicotine and Tobacco (SRNT) on the society's policies for disclosure of financial interests and potential conflicts of interest
2004 - 2008	Task Force on Medication Development, American College of Neuropsychopharmacology (ACNP). <u>Chair</u> of the subcommittee "Animal models and their validity to the disease disorder"
2006	Honorific Awards Committee, American College of Neuropsychopharmacology (ACNP)
2006	NIH Preclinical Treatment Units for Research on Neurocognition in Schizophrenia (TURNIS) subcommittee on animal models
2007 – Present	Credentials Committee, American College of Neuropsychopharmacology (ACNP)
2007 – Present	National Institute on Drug Abuse Medications Development Program Scientific Workgroup

Committee memberships at the University of California, San Diego:

2006 – Present: **Member** of the Executive Committee of the Department of Psychiatry, School of Medicine, University of California, San Diego

2007 – Present: **Member** of the Electives Committee of the School of Medicine, University of California, San Diego

Consultations for Non-Profit Institutions/Organizations:

Invited participant, Expert Group Meeting on Drug Craving Mechanisms, World Health Organization (WHO) / United Nations International Drug Control Programme (UNDCP), Vienna International Center, Austria (January 1992)

Invited participant, Drug Craving roundtable discussion (chair: Alan Leshner, NIDA Director), National Institute on Drug Abuse (NIDA), Washington DC (1995)

Invited participant, Clinical Definition of Drug Craving workshop, National Institute on Drug Abuse (NIDA), Washington DC (May 1998)

Invited participant (title of presentation: Animal Models of Depression), Department of Neuropharmacology of The Scripps Research Institute and the Nervous System Department of Novartis Pharma, Inc. on Mental Health (joint retreat), Liestal, Switzerland (June 1999)

Invited participant, Working Group Meeting: Cognitive and Emotional Aspects of Parkinson's Disease, National Institute of Neurological Diseases and Stroke (NINDS), National Institute of Mental Health (NIMH) and National Institute of Aging (NIA), Washington DC (January 2001)

Consultation Group, Neuroscience of Addiction report, World Health Organization (WHO), New Orleans LA (November 2000) and Mexico City, Mexico (June 2002)

Commissioned chapter (2002) ("Comorbidity of drug abuse with mental illness provides insights into the neurobiological abnormalities that may mediate these psychiatric disorders"), World Health Organization Report on Drug Addiction (2004)

Invited panel member, Advisory Council Workgroup: Setting Priorities for the Basic Sciences of Brain and Behavior, National Institute of Mental Health (NIMH), Washington DC (January 13, February 3, March 31, 2004) [www.nihm.nih.gov/council/brainbehavioralscience.cfm]

Invited panel member, NIMH Council Workshop (meeting on advising NIMH about future activities and initiatives in hastening the availability of new treatments for mental illness; chair: Thomas Insel, Director of NIMH), National Institute of Mental Health, Washington DC (January 22, 2004)

Invited participant, FDA/NIMH Measurement and Treatment Research to Improve Cognition in Schizophrenia (MATRICS) Workshop on Clinical Trial Design for Neurocognitive Drugs for Schizophrenia, Food and Drug Administration, National Institute of Mental Health, Washington DC (April 23, 2004)

Invited key participant (asked to facilitate interaction in animal models workgroup), NIMH Measurement and Treatment Research to Improve Cognition in Schizophrenia (MATRICS) meeting: New approaches to assessing and improving cognition in schizophrenia, Potomac MD (September 9-10, 2004)

Commissioned section ("Brain: How do effects and changes in the brain lead to addiction?"), Surgeon General's 2006 Report ("How Tobacco Causes Disease – The Biology and Behavioural Basis for Tobacco-Attributable Disease") (January-September 2005)

Invited participant, Biological Basis for Co-Occurrence of Substance Abuse and Other Psychiatric Disorders conference, National Institute on Drug Abuse (NIDA), Washington DC (April 2006)

Invited speaker and participant, Translational Medication Development for Nicotine Dependence Workshop, National Cancer Institute (NCI) / National Institute on Drug Abuse (NIDA), Washington DC (January 2007)

ORGANIZATION AND CHAIRING OF SYMPOSIA AND WORKSHOPS AT SCIENTIFIC MEETINGS

Co-organizer and moderator, Animal Models in Psychiatry (workshop), Society of Biological Psychiatry, 52nd meeting, San Diego CA (May 1997)

Co-chair and speaker (title of presentation: “Antidepressants and psychostimulant withdrawal: monoaminergic modulation of reward deficits”), Behavioral, Molecular, and Neurochemical Aspects of Stimulant Withdrawal in Rodents: Are There Similarities with Depression? (symposium), American College of Neuropsychopharmacology (ACNP), 40th Annual Meeting, Hawaii (December 2001)

Chair and speaker (title of presentation: “The role of metabotropic glutamate receptors in nicotine reinforcement and dependence”), The Role of Metabotropic Glutamate Receptors in Drug Dependence and Schizophrenia (symposium), Winter Conference on Brain Research (WCBR), Annual Meeting, Snowbird UT (January 2003)

Chair and speaker (title of presentation: “Drug withdrawal as an animal model of depression”), Animal Models of Depression: Recent Findings (symposium), International Behavioral Neuroscience (IBNS), Annual Meeting, San Juan, Puerto Rico (April 2003)

Invited chair, Addiction and Drugs of Abuse: Cocaine X (slide session), Society for Neuroscience (SFN), 33rd Annual Meeting, New Orleans LA (November 2003)

Invited chair, Oral Presentations in Basic Science, European Society for Research on Nicotine and Tobacco (SRNT), 5th Annual Meeting, Padua, Italy (November 2003)

Invited organizer, co-chair and speaker (title of presentation: “Animal models of emotion”), Starting-up Neurobiological Research on Nicotine and Tobacco: Course on Experimental Approaches and Methodologies (half-day Post-Graduate Course), European Society for Research on Nicotine and Tobacco (SRNT), 5th Annual Meeting, Padua, Italy (November 2003)

Invited to organize, moderate and present (title of presentation: “Role of GABA and Metabotropic Glutamate Receptors in Nicotine Dependence Processes: Potential Pharmacotherapies for Smoking Cessation”), Nicotine Dependence Treatment: New Perspectives (workshop/symposium), Tobacco-Related Disease Research Program (TRDRP) Annual Investigator Meeting (AIM2003), San Diego CA (December 2003)

Chair and speaker (title of presentation: “Behavioral and neurobiological mechanisms underlying nicotine addiction”), Neurobiology of Nicotine Addiction: From Rodent Molecules to Human Genetics (symposium), American College of Neuropsychopharmacology (ACNP), 42nd Annual Meeting, San Juan, Puerto Rico (December 2003)

Chair and speaker (title of presentation: “Nicotine withdrawal and craving”), The Neural Basis of Craving and Withdrawal in Drug Addiction (symposium), American College of Neuropsychopharmacology (ACNP), 42nd Annual Meeting, San Juan, Puerto Rico (December 2003)

Invited organizer and chair, Glutamate and Addiction (symposium), 5th Pharmacology, Biochemistry and Behavior Morzine Meeting, Morzine, Haute-Savoie, France (January 2004)

Invited chair and speaker (title of presentation: “Glutamatergic mechanisms underlying nicotine addiction”), New Molecular Insights into Addiction (symposium), 17th European College of Neuropsychopharmacology (ECNP), Annual Meeting, Stockholm, Sweden (October 2004)

Chair and speaker (title of presentation: “Metabotropic glutamate receptors and nicotine dependence”), Non-Nicotinic Therapies for Smoking Cessation (panel), American College of Neuropsychopharmacology (ACNP), 43rd Annual Meeting, San Juan, Puerto Rico (December 2004)

Chair and speaker (title of presentation: “Measuring anhedonia in animals: Reversal by antidepressant treatments”), Towards Building Translational Measures of Depression: Objective Characterizations of

Depressive Phenotypes and Biomarkers in Humans and Animals (panel), American College of Neuropsychopharmacology (ACNP), 44th Annual Meeting, Hawaii, U.S.A. (December 2005)

Chair, Development of Novel Metabotropic Glutamate Receptor Agents for the Treatment of Schizophrenia and Anxiety-related Disorders (panel), American College of Neuropsychopharmacology (ACNP), 44th Annual Meeting, Hawaii, U.S.A. (December 2005)

Invited to organize chair and present (title of presentation: "Role of mGluR5 and NMDA receptors in the reinforcing effects of nicotine"), Nicotine, Drugs of Abuse and Addiction (symposium), 6th Pharmacology, Biochemistry and Behavior Morzine Meeting, Morzine, Haute-Savoie, France (January 2006)

Chair and speaker (title of presentation: Glutamate receptors in primary reward and reward enhancing effects of nicotine), Glutamate and Reward Processes (panel), American College of Neuropsychopharmacology (ACNP), 46th Annual Meeting, Boca Raton, Florida, U.S.A. (December 2007)

Chair and discussant, Preclinical research on the discovery of glutamatergic therapeutics for the treatment of addiction (symposium), College on Problems of Drug Dependence (CPDD), 70th Annual Meeting, San Juan, Puerto Rico, U.S.A (June 2008)

PUBLICATIONS AND INVITED PRESENTATIONS

(Available upon request)

Unveiling the Functions of Presynaptic Metabotropic Glutamate Receptors in the Central Nervous System

DARRYLE D. SCHOEPP

Neuroscience Research Division, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana

Received April 3, 2001; accepted May 5, 2001 This paper is available online at <http://jpet.aspetjournals.org>

ABSTRACT

Metabotropic glutamate (mGlu) receptors, which include mGlu1–8 receptors, are a heterogeneous family of G-protein-coupled receptors which function to modulate brain excitability via presynaptic, postsynaptic and glial mechanisms. Certain members of this receptor family have been shown to function as presynaptic regulatory mechanisms to control release of neurotransmitters. In general, Gi-coupled mGlu receptor subtypes appear to negatively modulate excitatory (and possibly also inhibitory) neurotransmitter output when activated. Localization studies have shown that mGlu7 is restricted to the presynaptic grid at the site of vesicle fusion. These studies along with other evidence suggest that mGlu7 is the nerve terminal autoreceptor that regulates physiological release of glutamate. Other mGlu subtypes, in particular mGlu2, mGlu8,

and possibly mGlu4, are also localized presynaptically, but at perisynaptic sites outside the active zone of neurotransmitter release. Gi-coupled mGlu receptors also may exist on presynaptic elements of neighboring γ -aminobutyric acid (GABA) neurons where they play a role in heterosynaptic suppressions of GABA release. This suggests that these receptors may have evolved to monitor glutamate that has “spilled” out of the synapse. Thus, they may serve as the brain’s evolutionary mechanism to prevent pathological changes in neuronal excitability and thus maintain homeostasis. Recent progress on the molecular and pharmacological aspects of these presynaptic mGlu receptors is unveiling their functions and the therapeutic directions of agents designed for these novel glutamate receptor targets.

In the past decade there has been considerable progress in the field of metabotropic or G-protein-coupled glutamate (mGlu) receptors. For the most part, the cloning and identification of a novel heterogeneous family of mGlu receptors has driven this progress. There are currently eight known subtypes of mGlu receptors, which have been classified into three groups (see Table 1). Members of the mGlu receptor family are each G-protein-coupled receptors (GPCRs). Within each mGlu receptor group, there is ~70% sequence homology, whereas between the mGlu receptors subgroups there is lesser (~40%) homology. Group I mGlu receptors include mGlu1 and mGlu5, which when expressed are coupled via Gq to phospholipase C. Group II (mGlu2 and mGlu3) and group III (mGlu4,6,7,8) receptors are coupled to Gi and inhibit stimulated cAMP formation when expressed in cell lines. A number of gene splice variants for group I and III mGlu receptors are also

known, with most amino acid changes in the carboxyl-terminal regions that may be important in targeting receptors to regions of the cell (Boudin et al., 2000). These receptors each have unique but overlapping distributions in the central nervous system, and the functions of each subtype within these groups have recently been an active area of neuroscience research. In general, mGlu receptors appear to have evolved as modulatory mechanisms to control CNS excitability. Many disorders of the central nervous system, including psychiatric as well as neurological, have been linked to alterations in neuronal excitability via the glutamatergic system (Danysz et al., 1995). Thus, understanding ways to modulate CNS excitability by glutamate receptor mechanisms has broad therapeutic significance. In particular, certain members of the group II and group III mGlu receptors have been implicated in presynaptic negative modulation of excitatory glutamate and/or

ABBREVIATIONS: mGlu, metabotropic glutamate; GPCRs, G-protein-coupled receptors; CNS, central nervous system; GABA, γ -aminobutyric acid; EPSP, excitatory postsynaptic potential; MCPG, α -methyl-carboxyphenylglycine; 5HT, serotonin; L-AP4, L-2-amino-4-phosphonobutyric acid; 3,4-DCPG, (S)3,4-dicarboxyphenyl glycine; AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDA, N-methyl-D-aspartate; nRT, thalamic reticular nucleus.

TABLE 1
Classification, functions, and pharmacology of mGlu receptors

Receptor	Coupling	Localizations and Functions	Pharmacological Tools ^a
Group I mGlu1	Gq/↑ PLC	Primarily postsynaptic localization to neurons. Activation enhances post-synaptic glutamatergic excitability. Positive modulation of glutamate and GABA transmission suggested. Relative receptor expression noted in cerebellum. Involved in mediating LTD and LTP in certain synapses. Knockout animals exhibit motor learning and spatial learning deficits. Knockdown (antisense)-treated animals have reductions in excitatory responses to C-fiber (pain) inputs.	Prototypic group I agonists: 3,5-DHPG quisqualate Selective mGlu1 antagonist: CPCCOEt (noncompetitive)
mGlu5	Gq/↑ PLC	Primarily postsynaptic localization to neurons and glial cells. Positive modulation of glutamate and GABA neuronal transmission suggested. High expression through forebrain regions and in spinal cord. Involved in mediating NMDA-dependent LTP in certain synapses (CA1). Knockout animals exhibit deficits in acquisition and retention of spatial learning and contextual fear information. Pharmacological studies (MPEP) suggests a role in pain and anxiety states.	Selective mGlu5 antagonist: MPEP (noncompetitive)
Group II mGlu2	Gi, Go/↓ A.C.	Localizations pre- and postsynaptic in neurons. Negative modulation of glutamate and GABA neuronal transmission suggested. Relatively high expression levels noted in rat forebrain regions. Role in modulation of multiple forebrain limbic circuits (e.g. hippocampus) known. Loss of mGlu2/3 agonist suppressions of glutamate release and hippocampal LTD noted in mGlu2 knockout animals. No behavioral consequences in knockout animals yet reported. Spatial memory normal. Pharmacological data with mGlu2/3 agonists suggest possible antianxiety, antipsychotic, and neuroprotectant properties.	Prototypic group II agonists: DCG-IV 2R,4R-APDC LY354740 LY379268 Prototypic group II antagonists: EGLU LY341495 LY307452
mGlu3	Gi, Go/↓ A.C.	Primary localization postsynaptic to neurons and in glial cells. Role in modulation of GABA neuronal transmission noted. Phenotype of knockout animals not yet reported. Possible role in processing of noxious sensory stimuli within cortical thalamic circuits noted.	
Group III mGlu4	Gi, Go/↓ A.C.	Localizations presynaptic and postsynaptic in neurons. High receptor levels noted in rat cerebellum. Negative modulation of glutamate and GABA neuronal transmission suggested. Phenotype of knockout animals suggests role in motor function and seizure states in animals.	
mGlu6	Gi, Go/↓ A.C.	Highly localized to retinal tissues to dendrites of ON bipolar cells, with very low expression noted in brain tissue. Knockout animals suggest primary role is processing of visual sensory information.	Prototypic group III agonists: L-AP4 L-SOP PPG
mGlu7	Gi, Go/↓ A.C.	Localizations presynaptic and postsynaptic in neurons. Targeted presynaptically to the active zone of glutamate release. Negative modulation of glutamate and GABA neuronal transmission suggested. Phenotype of knockout animals suggests a role on amygdala-dependent learning and fear responses. Developmentally delayed seizures noted in knockout animals suggest a primary role in negative modulation of physiological glutamate release and possible the etiology of epilepsy.	Prototypic group III antagonists: CPPG MAP4
mGlu8	Gi, Go/↓ A.C.	Localizations pre- and postsynaptic in neurons. Negative modulation of glutamate (and possibly GABA neuronal transmission suggested). Likely presynaptic L-AP4 mGlu receptor in lateral perforant and neonatal spinal cord preparations. Phenotype of knockout animals not yet reported.	3,4-DCPG is a highly selective mGlu8 receptor agonist

A.C., adenylyl cyclase; LTD, long-term depression; LTP, long-term potentiation; 3,5-DHPG, 3,5-dihydroxyphenylglycine; CPCCOEt, 7-hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester; MPEP, 2-methyl-6-(phenylethynyl)pyridine; DCG-IV (2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine; 2R,4R-APDC, 2R,4R-4-aminopyrrolidine-2,4-dicarboxylic acid; EGLU (S)-α-ethylglutamic acid; L-SOP, L-serine-O-phosphate; PPG, (RS)-4-phosphonophenylglycine; MAP4, (S)-α-methyl-2-amino-4-phosphonobutanoic acid; CPPG, (2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine; PLC, phospholipase C.

^a See Schoepp et al. (1999a) for a detailed review of pharmacological agents.

inhibitory GABA neuronal transmission, and that subject and its therapeutic implications are the focus of this article. For recent comprehensive reviews of other aspects of

mGlu receptors, see Anwyl (1999), Bockaert and Pin (1999), Bordi and Ugolini (1999), Pin et al. (1999), Schoepp et al. (1999a), and Cartmell and Schoepp (2000).

Evolution of mGlu Receptors May Implicate Them as an Important Mechanism for Modulation of Neuronal Excitability

Because of their general structure and related sequence homologies, mGlu receptors are classified as family 3 GPCRs, which also include GABA_B (or "metabotropic" GABA receptors), Ca²⁺-sensing receptors, and certain pheromone receptors (see Bockaert and Pin, 1999). All of these receptors have in common a large extracellular ligand recognition domain, seven transmembrane-spanning regions connected by three intracellular loops and three extracellular loops, and a number of conserved cysteine residues that may be involved in receptor conformation by the formation of possible intra- or intermolecular disulfide linkages. Recent data suggest that functional family 3 GPCRs exist in situ as either hetero- (GABA_B) or homodimers (Ca²⁺-sensing and mGlu receptors). Family 3 GPCRs, particularly mGlu receptors, GABA_B receptors, and Ca²⁺-sensing proteins, are each highly expressed in the nervous system of many species (as well as in peripheral tissues to various degrees) of mammals, *Drosophila*, and fish (for reviews see Marshall et al., 1999; Bockaert and Pin, 1999; Riccardi, 1999; Schoepp et al., 1999a; Couve et al., 2000). This receptor class has structural features (a bi-lobed structure with an open configuration in absence and closed configuration in the presence of ligand) reminiscent of bacterial periplasmic binding proteins that function to sense nutrients (including ions and amino acids) for cellular uptake. Thus, from an evolutionary perspective, family 3 GPCRs in general may have evolved from a common primordial function (possibly Ca²⁺ sensing, see Riccardi, 1999). During evolution, the structure and functions of different family 3 GPCR have obviously diverged. Nevertheless, as discussed below, in the case of GABA_B and mGlu receptors, certain related roles in nervous system function, namely presynaptic modulation of brain excitability, have been apparently retained.

GABA and glutamate, respectively, are the major inhibitory and excitatory neurotransmitter substances in the mammalian nervous systems. The balance of excitation/inhibition within neuronal circuits is highly dependent upon postsynaptic activation of ionotropic receptors for these ligands (GABA_A or AMPA/kainate/NMDA receptors). The related proteins, GABA_B and mGlu receptors, function in nervous tissues to recognize ("sense") these respective ligands, but they serve a more modulatory role in the control of excitation/inhibition. It is interesting to consider that GABA_B and mGlu receptors are more related in functional and structural terms to each other than they are to their respective ionotropic receptor proteins, whose functions they apparently evolved to modulate. Recent data suggest that certain GABA_B and mGlu receptors are expressed presynaptically on both GABA and glutamate neurons where they monitor neuronal "spill-over" of their respective ligands, and play a role in heterosynaptic depression of either glutamate or GABA release (see Isaacson, 2000). The differential expression of these two metabotropic receptor systems may be important in determining the relative contribution of excitation versus inhibition in physiological and pathological states involving many circuits of the CNS. In other words, metabotropic amino acid receptors may have evolved as a primary mechanism to modulate neuronal excitability (see Fig. 1). In the case of mGlu

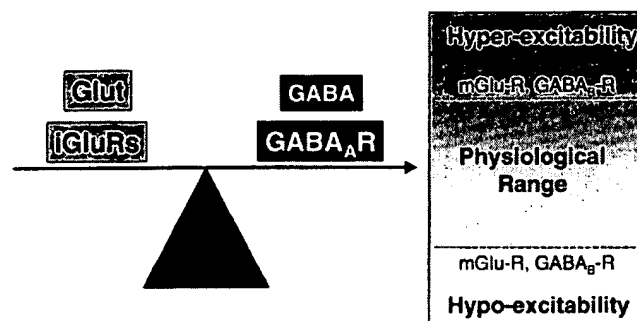


Fig. 1. Metabotropic glutamate and GABA_B receptors may have evolved to regulate neuronal excitability in the mammalian central nervous system. The amino acids L-glutamic acid (Glut) and GABA, respectively, are the primary excitatory and inhibitory neurotransmitter in the mammalian CNS. These substances mediate fast-synaptic transmission by activation of ionotropic glutamate receptors (iGluRs) and GABA_A receptors on postsynaptic neurons to control CNS excitability. In this manner, many physiological processes of the nervous system (motor, sensory, higher functions) are carried out. The modulatory roles of mGlu and GABA_B receptors (R) to control glutamate and GABA release have evolved as feedback monitors of glutamate and GABA levels within and outside the synaptic cleft. In this manner, these metabotropic receptors may play a role preventing pathological conditions of hyperexcitability (too much glutamate or not enough GABA) or hypoexcitability (not enough glutamate or too much GABA) within specific synapses where the receptors are expressed.

receptors, new information on localization and pharmacology of mGlu subtypes is beginning to reveal interesting details and possible therapeutic implications of these modulatory functions.

There is biochemical and electrophysiological evidence for a role of groups I, II, and III in the modulation of glutamate release (Anwyll, 1999; Cartmell and Schoepp, 2000). For the most part, group I mGlu receptors are considered to be primarily postsynaptic in localization, where they function to enhance cellular excitability via interactions with other postsynaptic processes (e.g., ionotropic receptors, ion channels) (see Bordi and Ugolini, 1999). Immunocytochemical studies have not yet confirmed a presynaptic localization of a group I mGlu receptor, although some biochemical evidence exists for a presynaptic role (see Cartmell and Schoepp, 2000). Nevertheless, a variety of approaches indicates that certain group II and group III mGlu receptor subtypes predominate on presynaptic elements where they function to regulate the release of glutamate in functionally diverse ways. Because a comprehensive review of the therapeutic aspects of mGlu modulation is beyond the scope of this article, select examples of therapeutic insights that have been gained from recent work are presented here.

Modulation of Glutamatergic Functions by Group II mGlu Receptor Subtypes

Of the group II metabotropic receptor subtypes, most of the data supports the conclusion that mGlu2 receptors are localized to preterminal axons of glutamate neurons where they function as a negative feedback mechanism to suppress further release of glutamate. mGlu3 receptors are primarily present postsynaptically on neurons and expressed in glia, where their functional role is less clear. Many studies use available antibodies and pharmacological agents that are group-II-selective (target both mGlu2 and mGlu3), so in many cases a clear distinction between the relative contribu-

tions of each subtype is difficult. For example, studies have clearly established that agonists for mGlu2/3 receptors reduce postsynaptic glutamate excitatory postsynaptic potentials (EPSPs) via a presynaptic mechanism in many synapses (reviewed by Anwyl, 1999). However, the presynaptic localization of mGlu2 to glutamate preterminal axons has been most clearly defined within the hippocampal formation. Here mGlu2 immunoreactivity illustrates a primary localization to subfields corresponding to the medial perforant and mossy fiber pathways (Shigemoto et al., 1997). Radioligand binding of the mGlu2/3-selective ligands, including either [³H]LY354740 (agonist) or [³H]LY341495 (antagonist), also supports this localization of mGlu2 (Schauffhauser et al., 1998; Wright et al., 2001). Unilateral lesions of entorhinal cortex, which contains cell bodies of the perforant path neurons, lead to ipsilateral loss of mGlu2/3 immunoreactivity (Shigemoto et al., 1997) and [³H]LY354740 binding (Richards et al., 2000) within perforant path terminal fields of the hippocampus that is associated with mGlu2 receptor labeling. Experiments with mGlu2 knockout mice most clearly indicate that mGlu2 is a presynaptic modulatory receptor in the hippocampus. Unlike wild-type animals, knockout mice deficient in mGlu2 receptors showed no immunostaining of mGlu2 within stratum lucidum of CA3 (mossy fiber terminal field) or stratum lacunosum moleculare of CA1 (medial perforant path terminal field) of hippocampus (Yokoi et al., 1996). In addition, in mGlu2-deficient mice, long-term depression induced by low-frequency stimulation of the mossy fiber CA3 synapses was abolished. Thus, presynaptic mGlu2 receptors appear to be essential for inducing long-term depression at this synapse, illustrating an important role in the modulation of neuronal excitability. However, that said, mGlu2 knockout animals appear to be functionally normal, with no alteration in basal synaptic transmission. This illustrates that mGlu2 receptors do not play a prominent role in acute regulation of excitatory synaptic transmission.

It is thought that following the release of glutamate, the levels in the immediate area of the synapse are likely in the millimolar range. Nevertheless, the binding affinity of the cloned mGlu2 receptor for glutamate is low micromolar (Schoepp et al., 1999a). This being the case, any mGlu2 receptors in the immediate vicinity of synapse might be partially occupied or even saturated with glutamate ligand. Although pharmacological data of glutamate release suppression by mGlu2/3 agonists support a presynaptic location of mGlu2 receptors, immunocytochemical studies do not support the presence of mGlu2 in the glutamate synapse per se. Antibodies selective for mGlu2 or mGlu2/3 receptors generally show immunolabeling to membrane compartments distant from active release sites and postsynaptic specializations (see Fig. 2). Although mGlu2/3 agonists have been shown to suppress glutamate release and postsynaptic excitations in a number of excitatory synapses (see Anwyl, 1999), it has also been observed that concentrations of antagonists per se that block this agonist effect have little effect on evoked excitatory synaptic transmission. Thus, presynaptic mGlu2 receptors that mediate agonist-induced negative feedback do not appear to be activated by endogenous glutamate to exert negative feedback under "normal" conditions of excitatory synaptic transmission. This raises questions about the role of these mGlu2 receptors in excitatory synaptic events.

Electrophysiology experiments support the concept that

synaptic spillover of glutamate is necessary for synaptic activation of mGlu2 receptor-mediated negative feedback on glutamate release. Scanziani et al. (1997) showed that the occupancy of presynaptic inhibitory (presumably mGlu2) receptors, in the rat mossy fiber pathway, was frequency-dependent. Enhanced excitatory synaptic responses were noted in the presence of the competitive mGlu2 antagonist α -methyl-carboxyphenylglycine (MCPG) under conditions of high (1-Hz) but not low (0.05-Hz) frequency stimulation. Moreover, glutamate uptake blockade with *L-trans*-pyrrolidine-2,4-dicarboxylic acid also produced decreases in field EPSPs, but only under conditions high-frequency stimulation. These data suggest that mGlu2 receptors may have evolved as a neuronal mechanism to keep glutamate transmission within the physiological range and thus prevent hyperexcitability from interfering with normal brain functions. Certain pharmacological studies with mGlu2/3 receptor agonists further support this hypothesis, as systemically active mGlu2/3 agonist compounds such as LY354740 and LY379268 are active in animal models of anxiety, global ischemia, and psychosis, at doses that have minimal or no effects on the animal's normal functions (Schoepp et al., 1999a,b). However, as discussed below, other factors may contribute to these observations.

In the rat prefrontal cortex, Marek et al. (2000) have demonstrated that mGlu2/3 agonists such as LY354740 and LY379268 suppress both electrically evoked and serotonin (5HT)-evoked EPSPs by a presynaptic mechanism. In this system, 5HT-evoked EPSPs, in contrast to electrically evoked EPSPs, appear to involve presynaptic, impulse-flow-independent release of glutamate that is mediated by 5HT_{2A} receptors. These actions appear to be presynaptic, as selective lesions of cell bodies in thalamic nuclei that project glutamatergic axons to medial prefrontal cortex lead to loss of 5HT_{2A} receptor-induced EPSPs (Marek et al., 2001). Here, the mGlu2 receptors that mediate these inhibitory effects appear to be tonically activated, as enhanced 5HT or electrically evoked excitatory synaptic responses were produced by the presence of an mGlu2/3 receptor antagonist (LY341495) per se. This indicates that conditions for the occupancy of presynaptic inhibitory mGlu2 receptors by endogenous glutamate may depend on the synapses involved. Also, in certain synapses such as the prefrontal cortex, mGlu2 receptor-mediated negative feedback appears to play a role in nonimpulse-flow (e.g., 5HT_{2A} receptor)-dependent regulation of glutamate release. Activation and antagonism of 5HT_{2A} receptors in the prefrontal cortex are important in mediating the actions of certain hallucinogens and antipsychotic drugs, respectively. The ability of mGlu2/3 (and group III) receptor agonists to act as a "functional" 5HT_{2A} antagonists (see Marek and Aghajanian, 1998) may have important therapeutic implications. Prefrontal cortex 5HT_{2A} receptor antagonism has been associated with the efficacious effects of atypical antipsychotic drugs, and within this area of the brain mGlu2/3 receptor agonists share the pharmacology in this functional sense.

Modulation of Glutamatergic Functions by Group III mGlu Receptor Subtypes

Koerner and Cotman (1981) initially described that the diacidic amino acid analog *L*-2-amino-4-phosphonobutyric acid (*L*-AP4) selectively suppressed glutamate excitations by

a presynaptic mechanism in the lateral perforant pathway of the hippocampus. This inhibitory activity of L-AP4 on glutamate excitations was also observed in other preparations, including the mossy fiber synapse, lateral olfactory tract, and spinal cord (see Thomsen, 1997). Until the 1990s, presynaptic inhibition induced by L-AP4 was ascribed to a relatively nebulous "L-AP4" receptor. However, with the cloning of the group III mGlu receptors, which Nakanishi (1992) defined by their sensitivity to L-AP4, it was recognized that certain group III mGlu subtypes might be responsible for L-AP4-induced suppression of glutamate release. Current data suggest a role for mGlu7, mGlu8, and possibly mGlu4 as candidates for these presynaptic effects of L-AP4 in the brain (see Thomsen, 1997).

In general, when compared with mGlu7 or mGlu2/3 receptors, the expressions of mGlu4 and mGlu8 receptors are somewhat more restricted in distribution in the CNS. Also, mGlu6 receptors have been shown in the retina, but they are not prominently expressed in the CNS. mGlu4 receptors are most prominently expressed in the cerebellum, where they have been studied in most detail, but they are also found in other brain and spinal regions to some extent (see Thomsen, 1997). Immunocytochemical studies indicate that mGlu4 receptors are expressed on presynaptic terminals and are present postsynaptically on neurons (Bradley et al., 1999). mGlu4 receptors are also suggested to be the peripheral taste receptors responsible for "unami" taste sensation to monosodium glutamate (Kinnamon and Margolskee, 1996). Within the cerebellum, localization and electrophysiological studies suggest that mGlu4 receptors mediate presynaptic inhibitory effects of L-AP4 on parallel fiber synapses to Purkinje cell dendrites. A presynaptic localization of mGlu4a receptors along the membranes of cerebellar parallel fiber terminals, with interspaced clusters of receptors along parallel fibers at intervals of 40 to 80 nM, was reported by Mateos et al. (1999).

Consistent with a role in modulation of parallel fiber-Purkinje cell synaptic transmission, mGlu4 receptor knockout mice have a loss of L-AP4-induced presynaptic inhibition of Purkinje cell synapses (Pekhletski et al., 1996). When compared with wild-type, these animals were deficient on a motor-learning test (rotarod), suggesting that expression of mGlu4 at parallel fiber-Purkinje cell synapses are important for normal motor function.

When compared with mGlu4 receptors, less is known about mGlu8 receptor distribution and functions, as fewer studies with antibodies to mGlu8 receptors have been reported, and the phenotype of mGlu8 receptor knockouts has not yet been described. In the rat and mouse, mRNA for the mGlu8 receptor is highly expressed in olfactory bulb (Duvoisin et al., 1995; Saugstad et al., 1997), suggesting that mGlu8 may be the "L-AP4" receptor responsible for presynaptic inhibition in the lateral olfactory tract. A presynaptic localization of mGlu8 receptors in projection neurons of the olfactory bulb in rats is supported by a study (Kinoshita et al., 1996) showing that transection of the lateral olfactory tract leads to decreases in mGlu8a immunoreactivity in layer 1a of the pyriform cortex (which is the target area for these glutamatergic projection neurons). The precise localization of mGlu8 receptors at the subcellular level is not yet clear. Electron microscopy studies show localization in close proximity to, but not necessarily within, the presynaptic specialization of asymmetrical synapses (Kinoshita et al., 1996). Like with mGlu2 receptors, glutamate has low micromolar affinity at mGlu8 receptors (Wright et al., 2000), possibly indicating a perisynaptic localization and similar functional role in extrasynaptic glutamate modulation of glutamate release (Fig. 2). mGlu8 receptors are also expressed to a lesser degree in other brain areas including cerebral cortex and cerebellum, but little is yet known of mGlu8 functions in these regions (Duvoisin et al., 1995; Saugstad et al., 1997).

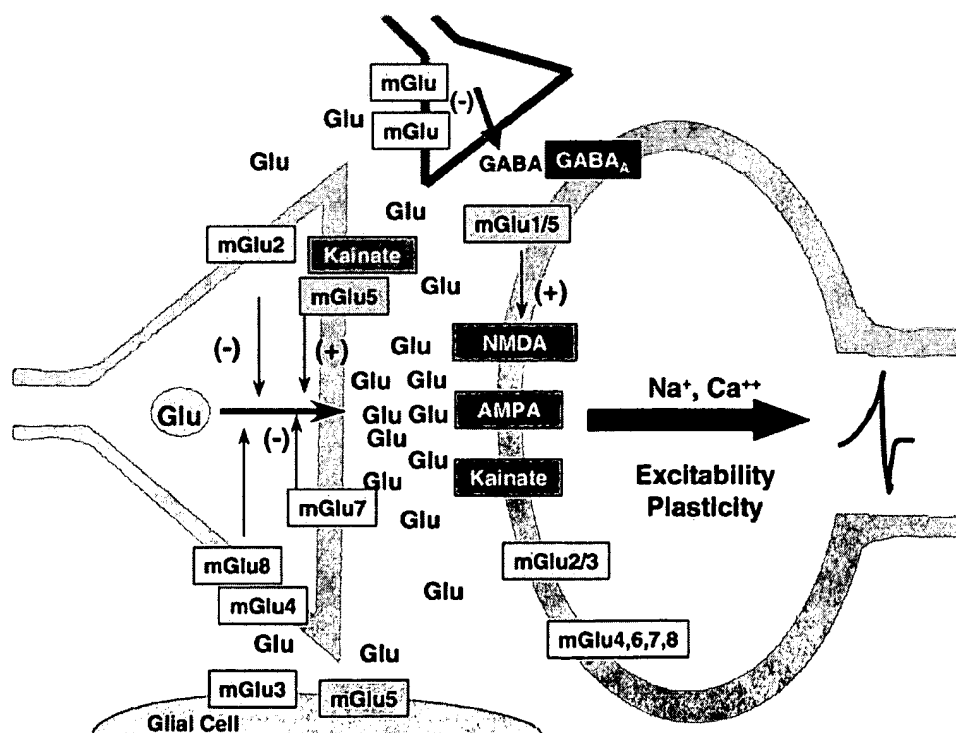


Fig. 2. General cellular localizations and cellular functions of mGlu receptor subtypes. In general, ionotropic glutamate receptors (AMPA, NMDA, and kainate subtypes) are postsynaptic where they function to mediate fast-excitations and synaptic plasticity associated with opening of sodium- and calcium-permeable ligand-gated ion channels. Metabotropic glutamate receptors are present at presynaptic, postsynaptic, glial, and heterosynaptic localizations where they function to monitor glutamate levels and provide positive feedback (group I mGlu receptors mGlu1/5) or negative feedback (group II mGlu receptors, mGlu2/3; and group III mGlu receptors, mGlu4/6/7/8) to decrease further release of neurotransmitters or change postsynaptic excitability to glutamate. The differential expression of these subtypes to certain synapses, cells, and cellular compartments allows for "finer" control of excitations throughout the CNS. Thus, drugs acting on metabotropic receptors may have therapeutic potential to treat a wide range of neurological and psychiatric conditions involving altered excitability of circuits in the brain and spinal cord.

Both mGlu4 and mGlu8 receptors have been shown to be expressed to a certain extent within certain subfields of the hippocampus, and their possible role in synaptic transmission at hippocampal pathways has been studied in some detail (Bradley et al., 1996; Bradley et al., 1999; Shigemoto et al., 1997). Among the group III mGlu subtypes, mGlu8 receptors are pharmacologically distinguished by sensitivity to the antagonist MCPG (Saugstad et al., 1997; Schoepp et al., 1999a). L-AP4 presynaptic inhibition of evoked excitations in the lateral perforant pathway of the hippocampus is also blocked by MCPG, suggesting a role for mGlu8 receptors in that glutamatergic pathway. Consistent with this conclusion, immunocytochemical studies have shown selective labeling of mGlu8 receptors to the terminal fields of the lateral perforant pathway (CA3 stratum lacunosum moleculare), and loss of this labeling following perforant path lesions (Shigemoto et al., 1997). Very recently, the compound (S)-3,4-dicarboxyphenylglycine (3,4-DCPG) has been described as a potent and highly selective mGlu8 receptor agonist, with no activity at cloned mGlu4, mGlu6, or mGlu7 receptors at concentrations that fully activate cloned mGlu8 receptors (Thomas et al., 2001). 3,4-DCPG appears to activate mGlu receptors on primary afferent glutamatergic terminals in the neonatal spinal cord to suppress evoked excitations, suggesting a role for mGlu8 in modulation of spinal sensory inputs (Thomas et al., 2001). This new mGlu8 agonist should be useful to further explore mGlu8 receptor function in the brain.

While mGlu8 receptors are relatively restricted to the terminal subfields of the dentate gyrus, the initial work of Bradley et al. (1996) showed mGlu4a staining was in cell bodies and dendrites of pyramidal neurons, granule cells, and scattered interneurons throughout the hippocampus. However, later work by this group using a more specific antibody (Bradley et al., 1999) suggested a more limited distribution of mGlu4a within the hippocampus. Here high expression was noted in the molecular layer of the dentate gyrus, stratum moleculare of CA1 and stratum oriens of the CA3 area. Importantly, this immunoreactivity was not present in the mGlu4a knockout mouse. Within the basal ganglia, mGlu4a immunoreactivity was shown to be on presynaptic axonal elements of striatopallidal neurons, as quinolinic acid lesions of these neurons decreased mGlu4a receptor immunoreactivity in the globus pallidus. At the electron microscope level, mGlu4 receptors are found postsynaptically at asymmetrical (presumably glutamatergic) synapses, and presynaptic at both asymmetrical and symmetrical (presumably GABAergic) synapses (Bradley et al., 1999). Thus, mGlu4 receptors may have pre- and postsynaptic functions, and they may be involved in both homo- and heterosynaptic modulation in these brain regions. Experiments designed to examine hippocampal functions in mGlu4 receptor knockout mice suggest a role in the processing of spatial information. Although mGlu4 receptor mutants were not impaired in the water maze task, they exhibited enhanced performance in a spatial reversal learning task (Gerlai et al., 1998). This phenotype may have been due to a decrease in the animal's memory retention of the original platform localization, producing a shorter escape latency to find a new platform location. In any case, this illustrates a possible role for mGlu4 receptors in hippocampal processing of spatial information.

mGlu7 receptors are highly expressed throughout the fore-

brain, brainstem, and spinal cord regions of the CNS (Bradley et al., 1998). In particular, mGlu7 receptors may represent an autoreceptor in certain synapses that provide negative feedback to limit further release of glutamate under normal physiological conditions of excitatory synaptic transmission (see Fig. 2). In cells expressing recombinant human or rat mGlu7 receptors, multiple laboratories observed that almost millimolar concentrations of glutamate were required to functionally activate this receptor (e.g., as measured by suppression of stimulated cAMP formation) (see Schoepp et al., 1999a). This relative insensitivity to glutamate activation when expressed in cell lines called into question whether non-neuronal cells expressing mGlu7 receptors were coupled as effectively as they might be in their native environment. However, radioligand binding studies with [³H]LY341495 in human mGlu7 receptor-expressing cell membranes have shown that the affinity of mGlu7 for glutamate is also relatively low ($K_i = 869 \mu\text{M}$) (Wright et al., 2000). Shigemoto et al. (1996) showed that mGlu7a receptor immunoreactivity in the rat hippocampus was restricted to the presynaptic grid or site of vesicle fusion. The lower affinity of glutamate for mGlu7 receptors is consistent with its localization in the synaptic cleft and function as an autoreceptor. To exist as a dynamic regulator of physiological glutamate release, the mGlu7 receptor cannot be fully occupied under basal conditions. Conceptually, the attainment of millimolar concentrations of glutamate at the presynaptic grid upon release would then occupy mGlu7 sites and activate its regulatory functions to further limit glutamate exocytosis. In general, mGlu7 receptor protein and mRNA are relatively more widespread in distribution throughout the neuro-axis when compared with other presynaptic mGlu receptors (e.g., mGlu2, mGlu4, and mGlu8), possibly indicating a more prominent role in normal regulation of synaptic glutamate release. Nevertheless, like other mGlu receptors, mGlu7 expression is more concentrated in certain areas and appears to be specifically targeted to certain synapses. Thus, not all glutamatergic neurons appear to express (or need) an mGlu7 autoreceptor regulatory mechanism to maintain normal excitatory functions.

The expression of mGlu7 receptors in glutamatergic nerve terminals of the perforant path is supported by loss of mGlu7a immunoreactivity following lesions of entorhinal cortex (Shigemoto et al., 1997). Colchicine lesions of the dentate granule cells also produced loss of mGlu7 receptor immunoreactivity in the CA3 of the hippocampus, indicating a presynaptic role of mGlu7 in the mossy fiber pathway. Interestingly, terminals of pyramidal neurons, which were presynaptic to the population of interneurons expressing postsynaptic mGlu1 receptors, expressed ~10-fold higher levels of mGlu7 receptors when compared with terminals making synaptic contacts with other pyramidal neurons or interneurons. This suggests that mGlu7 receptors may regulate release of glutamate at certain synapses based on what other receptors are expressed postsynaptically. A recent study (Boudin et al., 2000) suggests that the targeting of the mGlu7a receptor to the presynaptic membrane is dependent upon binding to PICK1, a PDZ domain binding protein. The PDZ domain binding site for the mGlu7a receptor is within the extreme carboxyl terminus of the receptor, and this sequence appears to confer PICK1 binding and receptor targeting, as mGlu2, another presynaptic mGlu receptor, did not bind to PICK1 and an mGlu7a receptor mutant lacking crit-

ical amino acids led to lack of presynaptic receptor clustering in hippocampal neurons. A presynaptic localization of mGlu7a receptors has also been demonstrated on glutamatergic terminals of the corticostriatal pathway (Kosinski et al., 1999), and the mGlu7a receptor appears to be expressed postsynaptically on neurons within the striatum as well. These data suggest a prominent role for mGlu7 in the extra-pyramidal control of movement and possibly in the etiology of movement disorders.

Neuronal cell bodies of dorsal root ganglion neurons and their axon terminals within the dorsal horn of the spinal cord also express mGlu7 receptors (Ohishi et al., 1995). Here, loss of mGlu7 immunoreactivity following rhizotomy indicates a regulatory role of mGlu7 in control of excitatory sensory information at the level of the spinal cord. However, that said, mGlu7 receptor knockout animals did not exhibit any abnormalities in pain sensitivity (Masugi et al., 1999), and the possible role of mGlu7 receptors in sensory transmission of noxious and non-noxious stimuli remains to be determined.

The targeted disruption of mGlu7 receptor expression in mice does lend some additional support to the notion of an autoreceptor role for mGlu7 protein. mGlu7 receptor-deficient mice were reported to develop epileptic seizures at ~12 weeks of age (Masugi et al., 1999), possibly due to their inability to regulate synaptic levels of glutamate into adulthood. Interestingly, in young knockout mice (prior to developing seizures), there was noted a prominent loss of presynaptic mGlu7 receptors within the amygdala complex. Indeed, mGlu7 receptor-deficient animals exhibited deficits in fear responses (freezing behavior following foot shock) and conditioned taste aversion (avoidance of taste stimuli that was associated with a toxic effect) when compared with responses in wild-type animals. These data suggest a role of mGlu7 receptors in the animal's expression of amygdala-dependent aversion learning and the expression of amygdala-dependent fear responses.

Modulation of Inhibitory Neurotransmission by Group II/III mGlu Receptors

Extra-synaptic localization of both group II (mGlu2 and mGlu3) and group III (mGlu4, mGlu7, mGlu8) mGlu receptors to nonglutamatergic neurons has been described and suggests a possible presynaptic heteroreceptor role for these receptors. Indeed, electrophysiological and biochemical studies have shown that mGlu2/3 receptor agonists and the group-III-selective agonist L-AP4 will suppress the release of GABA from neurons (see Anwyl, 1999; Cartmell and Schoepp, 2000). mGlu2/3 receptor agonists and L-AP4 will reversibly reduce the amplitude of GABA-mediated inhibitory postsynaptic potentials in a number of tissues including cerebral cortex, hippocampus, thalamus, and spinal cord (Anwyl, 1999). Thus, empirically mGlu receptor-mediated presynaptic modulation of GABA release may be a mechanism for enhancing cell excitability. However, the overall effects of this modulation would be dependent on the circuits these inhibitory interneurons are involved in controlling. For example, in CA1 of the hippocampus, Semyanov and Kullman (2000) demonstrated that the group III mGlu receptor agonist L-AP4 depresses GABAergic inhibitory postsynaptic currents in interneurons to a greater extent than GABAergic

inhibitory postsynaptic currents in pyramidal neurons. The selective depression of GABAergic transmission to interneurons was enhanced by glutamate uptake blockade and was prevented by α -methylserine-O-phosphate, a group III receptor antagonist. These data indicate that glutamate spillover from excitatory terminals may selectively disinhibit these inhibitory interneurons (via a decrease in GABA release on to other interneurons), and thus in fact lead to an overall suppression of excitatory synaptic transmission.

The work of Mitchell and Silver (2000) indicates that the spillover of glutamate from mossy fiber terminals can activate presynaptic mGlu receptors on GABAergic nerve terminals, and this leads to inhibition of GABA release onto principal excitatory neurons. This effect was mimicked by the nonselective mGlu agonist (\pm)-1-aminocyclopentane-*trans*-1,3-dicarboxylic acid, thus the mGlu receptor subtype responsible for this effect is not known. In any case, this represents a heterosynaptic mechanism by which inhibitory interneurons sense excitatory activity of neighboring excitatory synapses. In this manner, the efficacy of the active excitatory fibers onto CA3 pyramidal cells can be enhanced by locally reducing GABAergic inhibition.

As another example, most data suggest that activation of group III mGlu receptors are neuroprotectant in animal models in vivo, presumably due to the presynaptic suppression of glutamate release and reduced excitotoxicity mediated postsynaptically via ionotropic receptors (see Nicoletti et al., 1996). However, using cultured mouse striatal GABAergic neurons (which express mGlu7 receptors), Lafon-Cazal et al. (1999) showed that activation of presumable mGlu7 receptors with L-AP4 inhibited GABA release and ultimately enhanced neurotoxicity induced by NMDA. Thus, this expression of mGlu receptors in heterologous synapses to suppress GABAergic transmission needs to be considered when targeting mGlu receptors to suppress brain excitations in pathological states. This factor may explain why systemic administration of mGlu2/3 receptor agonists such as LY354740 and LY379268 *per se* to rats is not associated with profound suppressions on normal brain excitability (as measured by glucose utilization) (Lam et al., 1999). Possibly, coincident heterosynaptic inhibition of GABA release by these agonists contributes to counter any direct decreases in excitatory synaptic transmission on principal cells. Thus, overall, the actions of these types of drugs may be dependent on the relative roles of mGlu receptors to modulate presynaptic suppressions of glutamate versus GABA release in that functional circuit.

For example, thalamic relay neurons make excitatory synaptic contacts with GABAergic cells of the thalamic reticular nucleus (nRT), and they have been shown to express mGlu3 and mGlu4a receptors (Neto et al., 2000a). Interestingly, mGlu4 receptor knockout mice were found to be resistant to absence seizures induced by systemic administration of the GABA_A receptor antagonists such as bicuculline (Snead et al., 2000). The injection of a mGlu4 antagonist into the nRT of normal animals mimicked the resistance to bicuculline-induced seizures seen in the mGlu4a knockout animal. Conversely, nRT injection of an mGlu4 agonist to wild-type mice exacerbated bicuculline-induced seizures. These studies suggest a role for mGlu4 receptor-mediated modulation of thalamocortical GABAergic functions and a possible role for mGlu4a in pathological states such as absence seizures. It is

also suggested that modulation of GABAergic neurotransmission by mGlu4 receptor antagonist drugs may be useful to treat absence seizures in humans.

Furthermore, the relative roles of GABAergic inhibition and glutamatergic excitation within the brain can be altered in pathological states, and this may play a role in determining the ultimate actions of mGlu-selective compounds. For example, group II mGlu receptor agonists have been shown to produce hyperpolarization of GABAergic cells of nRT (Cox and Sherman, 1999), presumably due to activation of mGlu3 receptors (as mGlu2 receptor mRNA is not expressed in these cells) (Neto et al., 2000a). Interestingly, the induction of monoarthritis in rats by unilateral injection of complete Freund's adjuvant into the animal's tibiotarsal joint has been shown to produce a time-dependent and regionally specific up-regulation of mGlu3 receptor mRNA in nRT (Neto et al., 2000b). The direct injection of the mGlu2/3 antagonist (2S)- α -ethylglutamic acid in the nRT attenuated arthritic behavioral scores in these animals (Neto and Castro-Lopes, 2000). These studies suggest a possible role for mGlu3 modulation of nRT GABAergic functions in the central processing of certain noxious sensory stimuli, and a possible therapeutic avenue of mGlu3-selective antagonist compounds to treat certain forms for chronic pain. This is an interesting new concept, as group II and III receptor agonists have anti-pain effects in certain models, presumably due to reductions in pathologically enhanced neuronal hyperexcitability (Neugebauer et al., 2000). Ultimately, the effects of systemic mGlu antagonism (e.g., mGlu3) need to be further explored to investigate the optimal in vivo receptor profile for producing an mGlu receptor active anti-pain drug.

Conclusions

The identification of multiple mGlu receptor subtypes via molecular techniques, along with rapid advances in knowledge of their regional, cellular, and subcellular localizations, is providing new insights into how neuronal cell excitability is modulated in pathological as well as physiological states in animals and humans. Pharmacological studies and experiments with transgenic animals are filling in details of the roles of specific mGlu receptor subtypes in specific synapses, circuits, and brain regions. Ultimately, another benefit from this work may also be the development of highly novel, safe, and effective pharmacological agents to treat a range of neurological and psychiatric disorders in humans.

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Involvement of metabotropic glutamate receptors in excitatory amino acid and GABA release following spinal cord injury in rat

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Abstract

Spinal cord injury (SCI) leads to an increase in extracellular excitatory amino acid (EAA) concentrations resulting in glutamate receptor-mediated excitotoxic events. The glutamate receptors include ionotropic (iGluRs) and metabotropic (mGluR) receptors. Of the three groups of mGluRs, group-I activation can initiate intracellular pathways that lead to further transmitter release. Groups II and III mGluRs function mainly as autoreceptors to regulate neurotransmitter release. In an effort to examine the role of mGluRs in the increase in EAAs following SCI, we administered AIDA, a potent group-I mGluR antagonist immediately after injury. To determine subtype specific roles of the group-I mGluRs, we evaluated EAA release following LY 367385 (mGluR1 antagonist) and MPEP (mGluR5 antagonist) administration. To evaluate group-II and -III mGluRs we administered APDC (group-II agonist) and L-AP4 (group-III agonist) immediately following injury; additionally, we initiated treatment with CPPG (group-II/-III antagonist)

and LY 341495 (group-II antagonist) 5 min prior to injury. Subjects were adult male Sprague–Dawley rats (225–250 g), impact injured at T10 with an NYU impactor (12.5 mm drop). Agents were injected into the epicenter of injury, amino acids were collected by microdialysis fibers inserted 0.5 mm caudal from the edge of the impact region and quantified by HPLC. Treatment with AIDA significantly decreased extracellular EAA and GABA concentrations. MPEP reduced EAA concentrations without affecting GABA. Combining LY 367385 and MPEP resulted in a decrease in EAA and GABA concentrations greater than either agent alone. L-AP4 decreased EAA levels, while treatment with LY 341495 increased EAA levels. These results suggest that mGluRs play an important role in EAA toxicity following SCI.

Keywords: excitotoxicity, metabotropic glutamate receptors, spinal cord injury.

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The initial damage caused by injury to the CNS is often limited in area (McIntosh *et al.* 1998). However, there is a progressive expansion of the damaged area over time due to secondary effects. A primary cause of secondary damage is excitatory amino acid (EAA) toxicity. Following injury (e.g. ischemia in the brain or mechanical injury to the spinal cord), brief, but large, amounts of glutamate and aspartate are released at the injury site (Panter *et al.* 1990; Liu *et al.* 1991; McAdoo *et al.* 1999). This rise in extracellular EAAs may be due to exocytosis, inhibited or reversed uptake, blood brain barrier breakdown, and cell lysis (McAdoo *et al.* 2000; Rothstein *et al.* 1996; Tanaka *et al.* 1997; Vera-Portocarrero *et al.* 1999). The high levels of extracellular EAAs set up an excitotoxic cycle in which extracellular glutamate activates glutamate receptors, leading to more glutamate release that activates more glutamate receptors. The glutamate receptors are divided into two major types: (i) ionotropic receptors (iGluR): NMDA, AMPA and kainate receptors, and (ii) metabotropic receptors: metabotropic

glutamate receptors (mGluR), groups I, II and III. Since the mGluRs are G-protein coupled, their activation can initiate

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Abbreviations used: ACSF, artificial cerebral spinal fluid; AIDA, 1-aminoinidan-1,5-dicarboxylic acid; APDC, (2*R*,4*R*)-4-aminopyrrolidine-2,4-dicarboxylate; L-AP4, L-2-amino-4-phosphonobutyric acid; BLA, basolateral amygdala; CPPG, (*R,S*)- α -cyclopropyl-4-phosphonophenylglycine; DAG, diacylglycerol; EAA, excitatory amino acid; iGluR, ionotropic glutamate receptor; LY, 341495 (α S)- α -amino- α [(1*S*,2*S*)-2-carboxycyclopropyl]-9*H*-xanthine-9-propanoic acid; LY 367385, 2-methyl-4-carboxyphenylglycine; MK-801, (*S,R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine; MPEP, 2-methyl-6-(phenylethynyl)-pyridine; mGluR, metabotropic glutamate receptor; PKC, protein kinase C; SCI, spinal cord injury.

numerous intracellular signaling pathways that have a variety of complex and long-lasting effects.

Metabotropic glutamate receptors are divided into three groups based on sequence homology, transduction mechanisms and pharmacological profiles: (i) group I consists of mGluR1 and mGluR5; (ii) group II consists of mGluR2 and mGluR3; and (iii) group III consists of mGluR4, mGluR6, mGluR7 and mGluR8. Splice variants have been found for mGluR1 (α, β, c, e) mGluR4 (a,b) and mGluR5 (a,b) (Conn and Pin 1997). All subtypes of mGluRs, except for mGluR6 and mGluR8, are found in the spinal cord (Duvoisin *et al.* 1995; Valerio *et al.* 1997; Berthele *et al.* 1999). Group-I mGluRs are coupled to phosphatidylinositol hydrolysis, which leads to increases in intracellular Ca^{2+} levels and activation of protein kinase C (PKC) via diacylglycerol (DAG) formation. Increases in free cytosolic Ca^{2+} and activation of PKC are components of pathways that lead to cell death (Choi 1992). Activation of presynaptic group-I mGluRs enhances the release of glutamate (Herrero *et al.* 1992), which is facilitated by a PKC-mediated inhibition of presynaptic K^+ channels (Pin and Duvoisin 1995). Evidence for group-I receptors' involvement in neurodegeneration comes from injections of (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (agonist at group-I mGluRs), which has neurotoxic effects in the rat hippocampus and caudate nucleus (McDonald and Schoepp 1992; Sacaan and Schoepp 1992; McDonald *et al.* 1993) and in models of global ischemia (Henrich-Noack and Reymann 1999; Pellegrini-Giampietro *et al.* 1999). The neurotoxic effects are blocked by (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (MK-801), a noncompetitive, open channel NMDA antagonist, suggesting NMDA receptor involvement. There appears to be a synergistic effect between mGluRs and iGluRs, where activation of mGluRs potentiates iGluR responses, which in turn potentiate mGluR activation (Gereau and Heinemann 1998; Alagarsamy *et al.* 1999). Additional evidence for involvement of group-I mGluRs in neurotoxicity is shown by a selective mGluR1 antagonist, 2-methyl-4-carboxyphenylglycine (LY 367385) having neuroprotective effects using *in vitro* and *in vivo* models of excitotoxic death (Bruno *et al.* 1999; Mills *et al.* 2001b). Antagonists to group-I mGluRs have been shown to improve recovery of compound action potentials following spinal cord injury (Agrawal *et al.* 1998). Thus, activation of group-I mGluRs may contribute to excitotoxicity that leads to cell death.

In contrast, activation of group-II and III mGluRs may attenuate neuronal death (Bruno *et al.* 1994, 1995; Buisson and Choi 1995; Orlando *et al.* 1995; Turetsky *et al.* 1995; Buisson *et al.* 1996). Bruno *et al.* (1994) have shown that the group-II agonist (2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine inhibits kainate and NMDA-induced neurotoxicity in cortical neurons. Furthermore, activation of group-II mGluRs attenuates neuronal injury *in vitro*,

improves behavioral recovery following traumatic brain injury (Allen *et al.* 1999), and is protective in global ischemia (Bond *et al.* 1999). Group-III mGluR activation attenuates post-traumatic neuronal death *in vitro* (Faden *et al.* 1997). Activation of presynaptic group-II and -III mGluRs depresses synaptic transmission in basolateral amygdala (BLA) neurons (Rainnie and Shinnick-Gallagher 1992), which is enhanced 30-fold in kindled BLA neurons (Neugebauer *et al.* 1997). The inhibition of neurotransmitter release by activation of presynaptic group-II and -III mGluRs may occur via G-protein-mediated inhibition of high voltage-activated Ca^{2+} channels (Trombley and Westbrook 1992; Sahara and Westbrook 1993; Chavis *et al.* 1994; Ikeda *et al.* 1995; Choi and Lovinger 1996) and may involve glial interactions (Bruno *et al.* 1997). Thus, activation of group-II and -III mGluRs may attenuate neuronal death following injury by inhibiting the excitotoxic cascade.

Recently it was demonstrated that a mGluR group-I selective antagonist, 1-aminoindan-1,5-dicarboxylic acid (AIDA), decreases extracellular glutamate concentrations and is neuroprotective in gray and white matter following spinal cord injury (SCI; Mills *et al.* 2000, 2001b). Here, we extend these studies to examine different dosing regimens of AIDA and to determine which group-I subtype, mGluR1 or mGluR5, mediates the increase in EAA release using the mGluR1 selective antagonist, LY 367385, and the mGluR5 selective antagonist, 2-methyl-6-(phenylethynyl)-pyridine (MPEP). Additionally, we examined the roles of group-II and -III mGluRs in EAA and GABA release following SCI by administering agonists for group-II (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate (APCD), and group III, L-2-amino-4-phosphonobutyric acid (L-AP4) immediately following SCI. To further evaluate group-II and -III mGluRs, we initiated treatment preinjury with the antagonists (R,S)- α -cyclopropyl-4-phosphonophenylglycine (CPPG; group-II/-III) and (α S)- α -amino- α -[(1S,2S)-2-carboxycyclopropyl]-9H-xanthine-9-propanoic acid (LY 341495; group II). Table 1 provides a summary of the compounds used and their actions.

Table 1 Summary of mGluRs and pharmacological agents

Group	Subtypes	Transduction mechanisms	Compounds	Actions
I	1	+ PLC	AIDA, LY 367385	Antagonists
	5	+ PLC	AIDA, MPEP	Antagonists
II	2,3	- AC	APDC	Agonist
			CPPG, LY 341495	Antagonist
III	4,6,7,8	- AC	L-AP4	Agonist
			CPPG	Antagonist

Materials and methods

Experimental animals

Subjects were male Sprague–Dawley rats, 225–250 g, obtained from Harlan Sprague–Dawley, Inc. and housed with a light/dark cycle of 12 h/12 h. Experimental procedures were in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Sixty-three rats were divided into 16 groups ($n = 3$ –5 each): injury only; injury + agent: vehicle (post injury: 2 μ L); vehicle (post injury: 4 μ L); vehicle (pre through post injury: 4 μ L), AIDA (10 nmol, 200 nmol), LY 367385 (200 nmol), MPEP (200 nmol), LY 367385 + MPEP (200 nmol each), APDC (500 nmol), L-AP4 (500 nmol, 1.0 μ mol), CPPG (10 nmol, 50 nmol), and LY 341495 (30 nmol, 100 nmol).

Injury production

Spinal cord contusion injury was produced as previously described (Gruner 1992; Huang and Young 1994; Hulsebosch *et al.* 2000). Briefly, subjects were anesthetized by an intraperitoneal injection of pentobarbital (40 mg/kg). Anesthesia was considered complete when there was no flexor withdrawal in response to noxious foot pinch. The subjects' backs were shaven, an incision made to expose the vertebral column, and laminectomies were performed to expose spinal segments T10–T11. Spinal cord injury was produced using the New York University (NYU) injury device. A 10-g weight, 2.0 mm in diameter, was dropped from 12.5 mm onto the exposed cord.

Microdialysis sampling

Microdialysis sampling was conducted as previously described (Liu and McAdoo 1993). Briefly, microdialysis fibers were prepared by coating dialysis fibers (Spectrum Laboratories, Rancho Dominguez, CA, USA) with a thin layer of silicone rubber except for a 2-mm dialysis zone. Fused silica tubes (Polymicro Technologies, Phoenix, AZ, USA) were glued into the ends of the microdialysis tubing just outside the spinal cord to provide inlet and outlet connections. Including their coating, individual fibers had an external diameter of 180 μ m. The microdialysis fiber was inserted laterally through the cord 0.5 mm from the caudal edge of the impact zone (1.5 mm from the epicenter of injury) with one end attached to a syringe pump containing artificial cerebrospinal fluid (ACSF; containing, in mM: 151.1 Na^{2+} , 2.6 K^{+} , 0.9 Mg^{2+} , 1.3 Ca^{2+} , 122.7 Cl^{-} , 21.0 HCO_3^{-} and HPO_4^{2-}). The ACSF was bubbled with 95% O_2 /5% CO_2 prior to each experiment to adjust the pH to 7.2. Samples were collected at the outlet end of the tubing in small plastic vials on ice. ACSF was pumped through the fiber for 1 h to allow insertion damage to subside; then three sequential 15 min samples were collected for baseline values. The cord was then injured and four sequential 15 min samples were collected followed by six sequential 30 min samples, yielding a total collection time of 4 h after injury. Following injury, the cord was covered with mineral oil maintained at 37–38°C. Body temperature was maintained at 37–38°C using a homothermic blanket system (Harvard Apparatus, Holliston, MA, USA). Naphthalene dicarboxaldehyde derivatives of amino acids were analyzed by HPLC using fluorescence detection as previously (Liu and McAdoo 1993).

Drug and vehicle administration

All compounds were obtained from Tocris (Ballwin, MO, USA), prepared in ACSF, and injected stereotactically into the epicenter of

impact through a 30-gauge needle coupled to a syringe pump (CMA/Microdialysis AB, Stockholm, Sweden), at a depth of 1 mm immediately following SCI (within 3 min). Two different dosing regimens of AIDA (pH 7.4) were used: (i) A total volume of 2 μ L of a 5.0-mm solution (10 nmol total) was injected during the first 5 min after injury. (ii) A total volume of 4 μ L of a 50-mm solution (200 nmol total) was injected during the first 20 min following injury. LY 367385 (pH 7.4) and MPEP (pH 7.3) were both administered by injecting 4 μ L of a 50-mm solution (200 nmol total). LY 367385 + MPEP (200 nmol each; pH 7.3) was administered by injecting 4 μ L of a solution that was 50 mm for each. APDC and L-AP4 (pH 7.4) were administered by injecting 4 μ L of a 125-mm solution (500 nmol total). Additionally, another group of animals received 4 μ L of a 250-mm solution of L-AP4 (1.0 μ mol total). LY 367385, MPEP, LY 367385 + MPEP, APDC, and L-AP4 were administered during the first 20 min following injury. CPPG (30 nmol or 50 nmol) and LY 341495 (30 nmol or 100 nmol) were injected in 4 μ L of ACSF over a 20-min interval starting 5 min prior to injury. The doses of AIDA selected were based on previous work demonstrating the effect of AIDA on glutamate release and its neuroprotective actions following SCI (Mills *et al.* 2000, 2001b). For LY 367385, MPEP, APDC, and L-AP4, we selected the lowest doses that gave the maximum behavioral and neuroprotective effects (Bruno *et al.* 1999, 2000; Chapman *et al.* 1999, 2000; Mills *et al.* 2001b,c). CPPG and LY 341495 doses were based on previous pharmacological studies (Jane *et al.* 1996; Kingston *et al.* 1998; Johnson *et al.* 1999; Naples and Hampson 2001).

One concern with dose selection, particularly with the group-I antagonists, is the interactions of these compounds with iGluRs. For example, MPEP has been reported to inhibit NMDA receptor activity at concentrations above 20 μ M in cultured rat cortical neuronal cells (Movsesyan *et al.* 2001), while other studies report no effects on NMDA receptors at 100 μ M in *Xenopus laevis* oocytes (Gasparini *et al.* 1999). LY 367385 affects iGluRs at IC_{50} values > 10 mM (Bruno *et al.* 1999); however, one study reports a slight enhancement of NMDA and AMPA responses after treatment with 50 mM of LY 367385 in the ventrobasal thalamus (Salt and Binns 2000). To calculate the maximum possible concentration of an agent at the microdialysis fiber, we assumed that the spinal cord could be approximated by a cylinder (radius of 1.5 mm and length 3.0 mm; injections were performed 1.5 mm from the dialysis fiber and assuming equal diffusion in both directions) and calculated its volume. The concentration of the agent was calculated assuming uniform diffusion throughout the cylinder. To illustrate, injection of 200 nmol of LY367385 would give a maximum concentration at the microdialysis fiber of 9.4 mM, which is below the concentrations reported to effect iGluRs (Bruno *et al.* 1999). It is important to note that actual concentrations of the agents at the microdialysis fiber are probably much less due to uptake and tortuosity (a measure of how diffusing molecules are hindered by cellular obstructions; Lieberman *et al.* 1995; Lonser *et al.* 1998; Nicholson 1999).

Statistical analysis

Glutamate, aspartate, and GABA concentrations were analyzed using a two-sample *t*-test with an α level set *a priori* at 0.05 at individual time points to test for significant differences between injury only, injury + vehicle, and injury + agent treatment groups.

When indicated, *post hoc* pairwise comparisons between treatment groups were made using Fisher's least significant difference (LSD) test. Data are expressed as means \pm SEM.

Results

Extracellular EAA and GABA concentrations following SCI

Following SCI there is an increase in extracellular glutamate and aspartate concentrations that reach maximum levels of $49.2 \pm 8.0 \mu\text{M}$ and $14.6 \pm 2.1 \mu\text{M}$, respectively, which slowly return to baseline levels by 120 min after injury (Figs 1a and b). Compared with injury alone, treatment with each vehicle (ACSF) regiment did not significantly affect extracellular glutamate or aspartate levels (Figs 1a and b). Normal basal levels of GABA in the spinal cord are extremely low (below $0.03 \mu\text{M}$; data not shown); however, following injury extracellular GABA concentrations rise to reach maximum levels of $4.8 \pm 0.8 \mu\text{M}$ and remain elevated through 30 min following injury ($1.1 \pm 0.4 \mu\text{M}$; Fig. 1c). By 45 min following injury extracellular GABA levels return to baseline levels. Therefore, GABA concentrations are reported for only the first 30 min following injury. Compared with injury alone, vehicle treatment (any volume or duration) did not affect extracellular GABA levels at any time point analyzed (Fig. 1c).

AIDA effects on extracellular EAA concentrations

Administration of 10 nmol of AIDA, a mGluR group-I selective antagonist, during the first 5 min of injury significantly reduced extracellular glutamate concentrations to $27.7 \pm 2.5 \mu\text{M}$ and aspartate concentrations to $4.7 \pm 1.1 \mu\text{M}$ (Figs 2a and c; $p < 0.05$). Increasing the dose of AIDA to 200 nmol, given over a 20-min period following SCI, did not result in a further decrease in extracellular glutamate or aspartate concentrations (Figs 2b and d). Maximum glutamate and aspartate concentrations following 200 nmol of AIDA were $32.9 \pm 6.0 \mu\text{M}$ and $6.6 \pm 1.8 \mu\text{M}$, respectively. Since both 10 and 200 nmol of AIDA produced similar effects, it is likely that saturation of group-I mGluRs occurs below 10 nmol of AIDA.

LY 367385 and MPEP effects on extracellular EAA concentrations

Administration of the mGluR1 specific antagonist LY 367385 (200 nmol over 20 min) did not affect the initial increase in extracellular glutamate or aspartate concentrations following SCI (Figs 3a and d). Extracellular glutamate and aspartate levels at 15 min following SCI were $44.9 \pm 4.9 \mu\text{M}$ and $11.8 \pm 1.6 \mu\text{M}$ in the LY 367385-treated group. Administration of the mGluR5 specific antagonist MPEP (200 nmol over 20 min) significantly reduced both glutamate and aspartate levels immediately following SCI (Figs 3b and e; $p < 0.05$). Extracellular

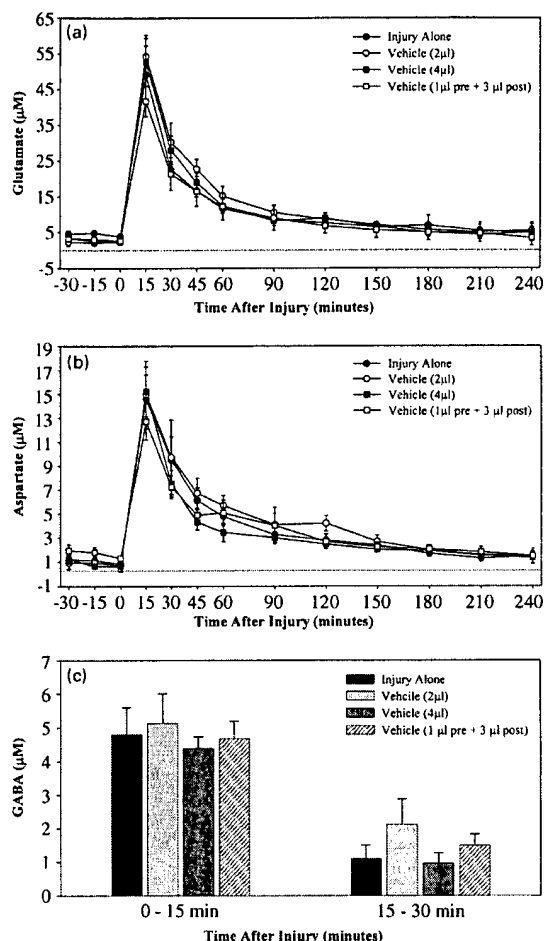


Fig. 1 Time course of extracellular glutamate (a), aspartate (b), and GABA (c) concentrations after SCI for injured only animals and each vehicle treatment. Vehicle treatments did not significantly affect extracellular glutamate, aspartate, or GABA concentrations after SCI. Data are means \pm SEM.

glutamate and aspartate levels at 15 min following SCI were $37.2 \pm 4.7 \mu\text{M}$ and $8.9 \pm 1.0 \mu\text{M}$ in the MPEP-treated group. When LY 367385 and MPEP were administered together, extracellular glutamate and aspartate concentrations were reduced further than when each agent was administered separately to $19.5 \pm 5.4 \mu\text{M}$ and $4.6 \pm 1.0 \mu\text{M}$, respectively (Figs 3c and f).

Group-I antagonists' effects on extracellular GABA concentrations

Following SCI, treatment with AIDA (10 and 200 nmol) significantly reduced extracellular GABA levels compared with injury alone and the vehicle-treated group within the first 15 min of injury (Fig. 4a; $p < 0.05$). Treatment with

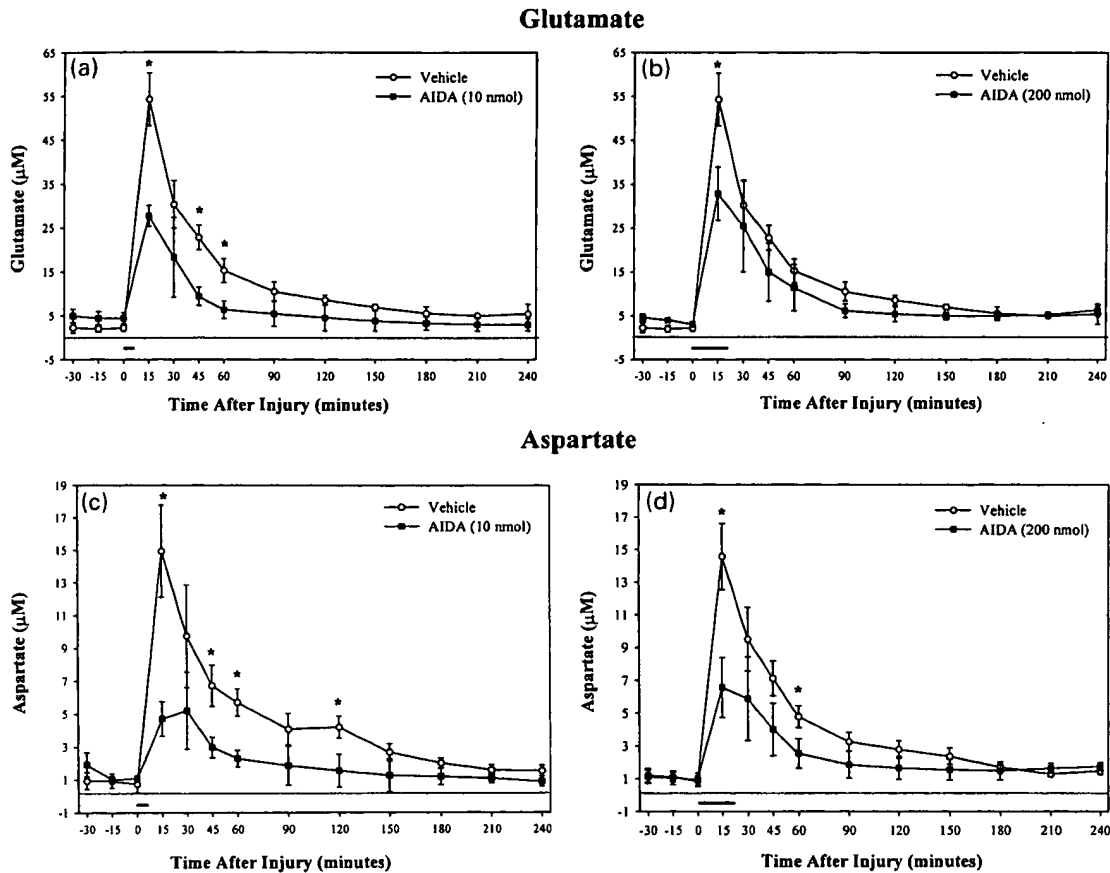


Fig. 2 Time course of extracellular glutamate (a and b) and aspartate (c and d) concentrations after SCI with 10 nmol (a and c) or 200 nmol (b and d) of AIDA. Following SCI, there is an increase in extracellular glutamate and aspartate concentrations that slowly returns to baseline values by 120 min after injury. Treatment with AIDA at 10 nmol (a) or 200 nmol (b) significantly reduced extracellular glutamate levels compared with the vehicle-treated group during

the first 15 min following SCI. Similarly, aspartate levels were decreased in the AIDA-treated group [10 nmol (c) and 200 nmol (d)] during the first 15 min after injury compared with the vehicle-treated group. Data are means \pm SEM. *Indicates a statistically significant difference between AIDA-treated and vehicle-treated ($p < 0.05$). Treatment duration is indicated by a horizontal line above the x-axis.

LY 367385 or MPEP did not affect extracellular GABA concentrations after SCI. However, combining LY 367385 and MPEP resulted in a significant decrease in extracellular GABA concentrations compared with injury alone and the vehicle-treated group within the first 15 min of SCI (Fig. 4a; $p < 0.05$). By 30 min after SCI, there was not a statistically significant difference between injury alone or the vehicle-treated group versus any group-I agent treated group (Fig. 4b).

APDC and L-AP4 effects on extracellular EAA and GABA concentrations

Following SCI, 500 nmol of APDC did not affect the initial rise in extracellular glutamate or aspartate concentrations

(Figs 5a and c). Glutamate and aspartate levels at 15 min following SCI were $37.8 \pm 7.3 \mu\text{M}$ and $12.7 \pm 2.4 \mu\text{M}$, respectively, in the APDC-treated group. Administration of L-AP4 (500 nmol) significantly reduced extracellular glutamate levels up to 30 min following SCI. Glutamate and aspartate levels at 15 min following SCI were $30.9 \pm 6.2 \mu\text{M}$ and $9.0 \pm 1.7 \mu\text{M}$, respectively, in the L-AP4 (500 nmol)-treated group (Figs 5b and d; $p < 0.05$). Since EC_{50} values for L-AP4 vary greatly between mGluR4 and mGluR7 subtypes (Conn and Pin 1997), we tested a higher dose of L-AP4 (1.0 μmol). Only a slight decrease, which was not statistically significant compared with 500 nmol of L-AP4, in extracellular glutamate and aspartate concentrations was seen (Figs 5b and d). Treatment with

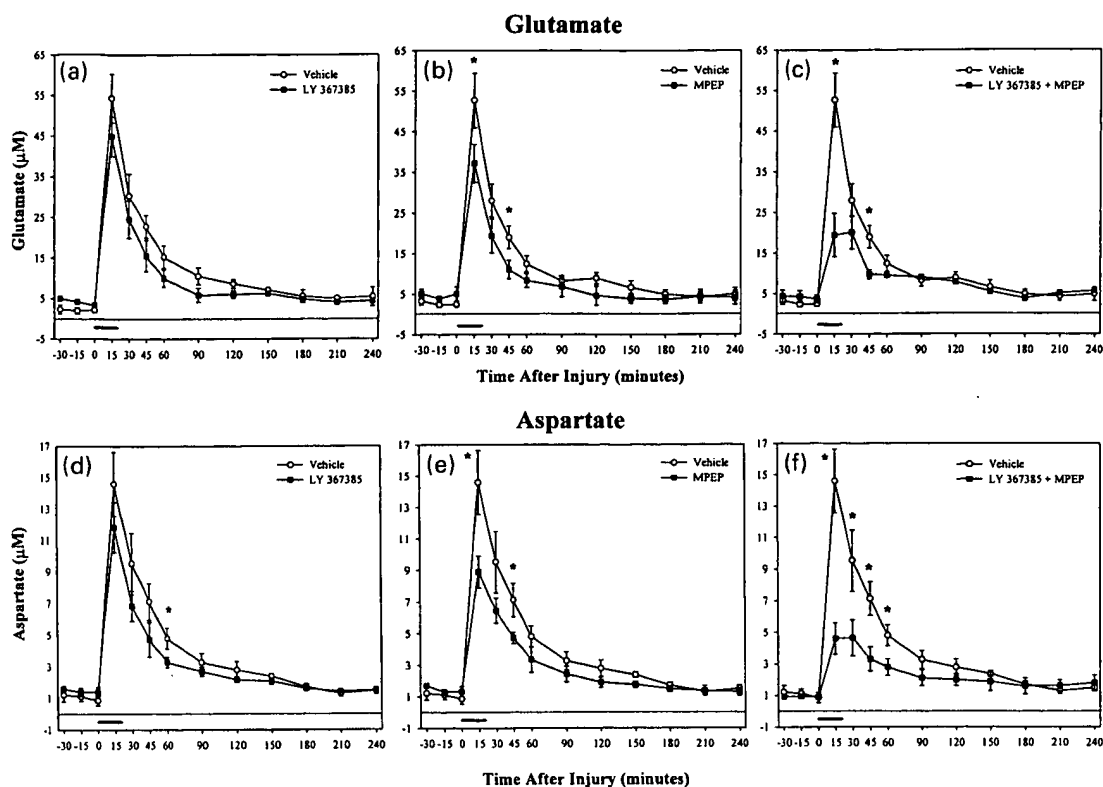


Fig. 3 Time course of extracellular glutamate (a–c) and aspartate (d–f) concentrations after SCI in LY 367385- (a and d), MPEP- (b and e), and LY 367385 + MPEP-treated (c and f) groups. MPEP (b), but not LY 367385 (a), reduced extracellular glutamate concentrations following SCI. However, combining LY 367385 and MPEP (c) resulted in a greater decrease in extracellular glutamate

concentrations than either agent alone. Similar results were obtained for aspartate levels following SCI for LY 367385 (d), MPEP (e), and LY 367385 + MPEP (f). Data are means \pm SEM. *Indicates a statistically significant difference between treatment groups ($p < 0.05$). Treatment duration is indicated by a horizontal line above the x-axis.

APDC and L-AP4 did not significantly affect extracellular GABA concentrations at any time point (Fig. 6a).

CPPG and LY 341495 effects on extracellular EAA and GABA concentrations

Treatment with CPPG (10 or 50 nmol) from 5 min prior through 15 min post injury did not have a significant effect on extracellular glutamate or aspartate levels (Figs 7a and c). However treatment with LY 341495 (30 and 100 nmol) from 5 min prior through 15 min after injury increased extracellular glutamate and aspartate levels 15–30 min post injury compared with vehicle control ($p < 0.05$; Figs 7b and d). Neither CPPG nor LY 341495 treatment affected extracellular GABA concentration following injury (Fig. 6b).

Discussion

The present study demonstrates that individual subtypes of mGluRs differentially influence extracellular EAA and

GABA concentrations following SCI. Within the group-I mGluRs, antagonist treatment to mGluR1 had less of an effect on extracellular EAA concentrations than did antagonist treatment against mGluR5. However, combining treatments had a greater effect than either treatment alone. Activation of group-III, but not group-II, mGluRs following SCI reduced extracellular EAA levels without affecting GABA concentrations. Antagonist treatments to group-II mGluRs initiated prior to injury increased EAA concentrations above injury-induced values. These results suggest that mGluRs play important roles in the initial events that lead to excitotoxicity following CNS injury.

Group-I mGluRs and EAA release

Activation of group-I mGluRs enhances neurotransmitter release (Herrero *et al.* 1992), which is facilitated by a PKC-mediated inhibition of presynaptic K^+ channels (Pin and Duvoisin 1995). The activation of group-I mGluRs may increase EAA release by modulating the membrane

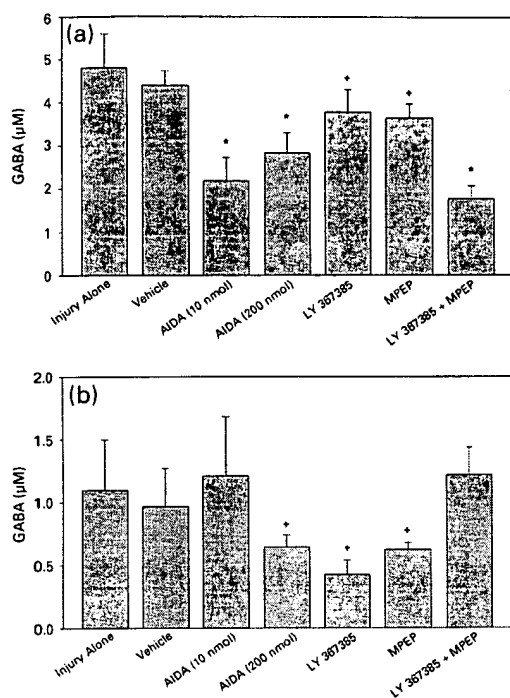


Fig. 4 Extracellular GABA concentrations 0–15 min (a) and 15–30 min (b) following SCI. Treatment with AIDA (10 and 200 nmol) and LY 367385 + MPEP reduced extracellular GABA concentrations immediately following SCI compared with the vehicle-treated group (a). However, 15–30 min after SCI, no treatment group was significantly different from the vehicle-treated group (b). Data are means \pm SEM. *Indicates a statistically significant difference compared with vehicle-treated; + indicates a statistically significant difference compared with LY 367385 + MPEP ($p < 0.05$).

potential through inhibition of IK_{leak} and IK_M or increase the firing rate by inhibiting IK_{AHP} (Pin and Duvoisin 1995). A previous report showed that the group-I antagonist AIDA reduced extracellular glutamate concentrations following SCI (Mills *et al.* 2000). The present study extends these results to demonstrate that higher doses of AIDA do not further reduce EAA levels following SCI. Since AIDA antagonizes both mGluR1 and mGluR5 (Schoepp *et al.* 1999), its effects do not reveal which group-I subtype, mGluR1, mGluR5, or both, mediates these responses.

To test involvement of individual group-I subtypes in EAA release following SCI, we used the mGluR1 specific antagonist LY 367385 and the mGluR5 specific antagonist MPEP. MPEP, but not LY 367385, significantly decreased glutamate and aspartate extracellular concentrations immediately following SCI. This suggests that mGluR5 plays a greater role than mGluR1 in modulating the increase in extracellular glutamate and aspartate concentrations after SCI. Group-I mGluRs can potentiate iGluR responses and

iGluRs can facilitate mGluR activation (Aniksztejin *et al.* 1992; Bleakman *et al.* 1992; Kelso *et al.* 1992; Glaum and Miller 1993; Ugolini *et al.* 1997, 1999; Budai and Larson 1998; Alagarsamy *et al.* 1999; Salt and Binns 2000). Inhibition of this synergistic relationship is one mechanism by which antagonism of group-I mGluRs may affect neurotransmitter release. Since MPEP, but not LY 367385, inhibited glutamate release, it may be that mGluR5 is more tightly coupled than mGluR1 to iGluR potentiation in the spinal cord.

Group-I mGluRs have been shown to inhibit or increase transmitter release depending on neuronal activity (Herrero *et al.* 1998; Rodriguez-Moreno *et al.* 1998; Sistiaga and Sanchez-Prieto 2000). A functional switch that is mediated by receptor phosphorylation has been proposed to account for these conflicting actions (Herrero *et al.* 1998). Under basal conditions, activation of group-I mGluRs facilitates release, whereas activation during times of excess extracellular glutamate inhibits release (Herrero *et al.* 1998). If mGluR1 undergoes the switch from facilitation to inhibition while mGluR5 remains in a facilitatory state, mGluR1 antagonists would not have as great of an effect as mGluR5 antagonists on transmitter release, consistent with observations in the current study. Expression levels of mGluR5 are much higher than those of mGluR1 in the rat thoracic spinal cord (Mills *et al.* 2001a). Thus, a simpler explanation is that antagonism of mGluR5 has a greater effect simply because there is more mGluR5 than mGluR1 in the rat thoracic spinal cord.

However, when antagonists for each subtype were combined, a greater decrease in extracellular EAAs was seen than when agents were tested individually. The different subcellular localizations of mGluR1 and mGluR5 in the spinal cord (Alvarez *et al.* 2000) may explain why a combination of mGluR1 and mGluR5 antagonists is more effective than the individual treatments. mGluR5 appears to be targeted to distal dendritic regions (except in lamina II), whereas mGluR1 is seen throughout the somatodendritic membrane (Alvarez *et al.* 2000). Furthermore, mGluR5 is more highly expressed than mGluR1 in the rat thoracic spinal cord (Mills *et al.* 2001a). Thus, there may be more mGluR5 in synaptic zones, while the less expressed mGluR1 is more dispersed. Therefore, antagonists against mGluR5 would be expected to have a greater effect than mGluR1 antagonists, and mGluR1 treatments might not produce an observable effect on EAA release. Combining the two treatments would affect both the large population of mGluR5s in the dendritic zones (directly affecting EAA release) and the mGluR1s in the cell soma (affecting membrane potential), which would produce a larger effect than either treatment alone. To our knowledge there is currently no study that specifically addresses mGluR1 effects on mGluR5 responses or mGluR5 effects on mGluR1 responses that would help assess this possibility.

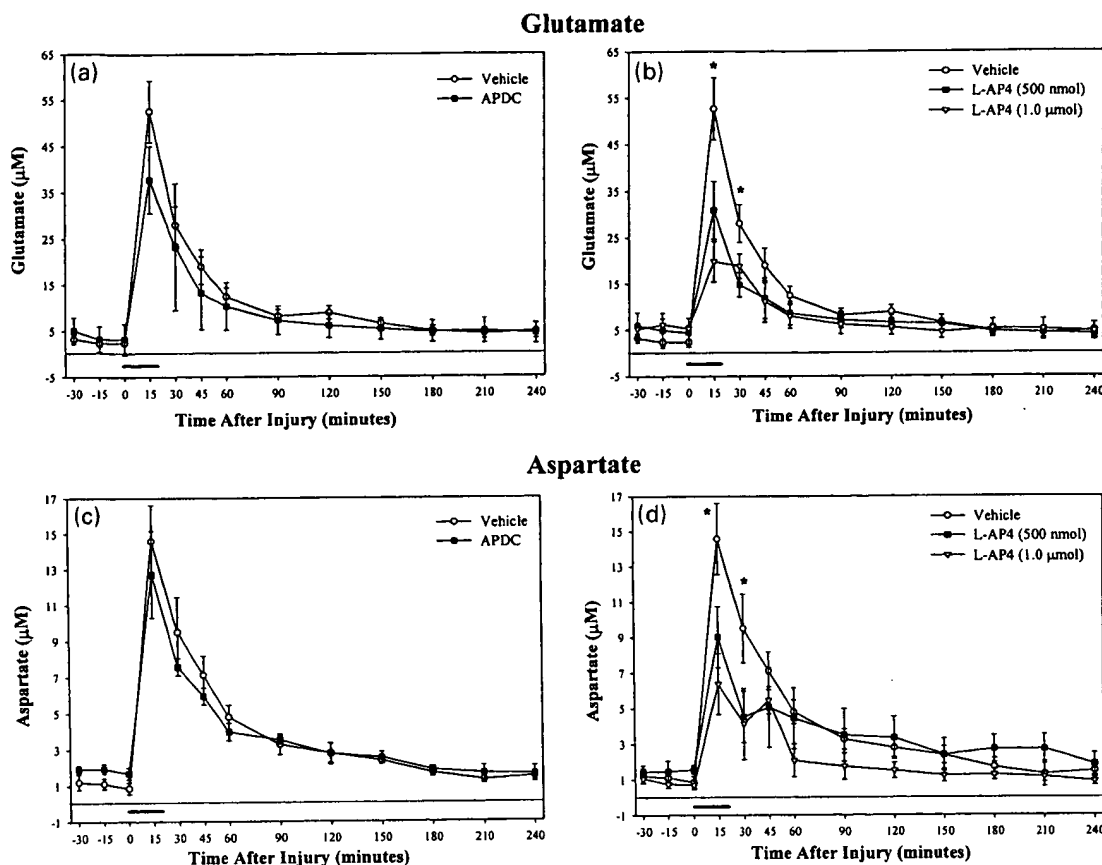


Fig. 5 Time course of extracellular glutamate (a and b) and aspartate (c and d) concentrations after SCI in APDC- (a and c) or L-AP4-treated (b and d) groups. L-AP4, but not APDC treatment decreased extracellular glutamate and aspartate concentrations following SCI.

Data are means \pm SEM. *Indicates a statistically significant difference between the vehicle and treatment groups ($p < 0.05$). Treatment duration is indicated by a horizontal line above the x-axis.

Group-II and III mGluRs and EAA release

The role of group-II and -III mGluRs as autoreceptors to regulate neurotransmission in the CNS is well established. The primary mechanism by which group-II and -III mGluRs regulate neurotransmitter release is presumably through inhibition of Ca^{2+} channels (Pin and Duvoisin 1995). However, other mechanisms of modulating neurotransmission independent of Ca^{2+} channel modulation have been suggested. For example, mGluR autoreceptors may affect presynaptic release machinery (Bushnell *et al.* 1999; Gerber *et al.* 2000). In autaptic hippocampal cultures, mGluR7 is colocalized with synaptophysin, suggesting a modulation of neurotransmitter release that is 'downstream' of Ca^{2+} entry (Bushnell *et al.* 1999).

Allen *et al.* (1999) demonstrated that the group-II agonist LY 354740 attenuates injury-induced glutamate release in neuronal-glial cultures at 2 h post injury. However, in the

current study the group-II agonist APDC did not reduce extracellular EAA concentrations following SCI. In the spinal cord, group-II and -III mGluRs are found presynaptically (Ohishi *et al.* 1995; Azkue *et al.* 2000, 2001). Group-II mGluRs appear to be localized in the preterminal area removed from transmitter release sites (Azkue *et al.* 2000; Shigemoto *et al.* 1997), whereas mGluR4a (group-III mGluR) is clustered along the presynaptic specialization (Azkue *et al.* 2001). The lack of effect of APDC on extracellular EAA levels may be due to the cellular localization of group-II mGluRs in the adult rat spinal cord (farther from the synapse than group III). Excitatory, inhibitory, dual, and mixed effects of group-II mGluRs have been found in the spinal cord (Bond and Lodge 1995; Cao *et al.* 1995, 1997; King and Liu 1996; Bond *et al.* 1997; Stanfa and Dickenson 1998; Dong and Feldman 1999). Thus, another possibility is that activation of spinal group-II

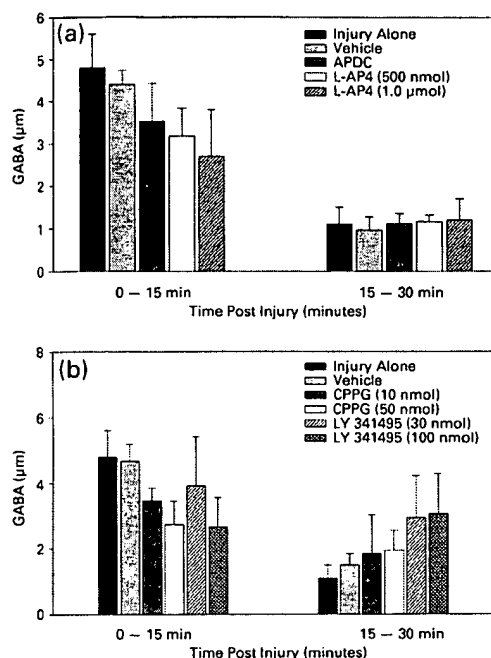


Fig. 6 Extracellular GABA levels after postinjury treatment (0–20 min) following administration of vehicle, APDC, or L-AP4 (a) and treatment initiated pre-injury (–5–15 min) with vehicle, CPPG, or LY 341495 (b). Post-injury treatment with agonists to group II (APDC) or group III (L-AP4) did not affect extracellular GABA concentrations during the first 30 min of injury. Treatment initiated pre-injury with antagonists to group II (CPPG) or group III (LY 341495) did not affect extracellular GABA concentrations during the first 30 min after injury. Data are means \pm SEM.

mGluRs has both inhibitory and excitatory effects, producing an end result of no overall effect (Neugebauer *et al.* 2000).

Interestingly, treatment 5 min prior through 15 min post-SCI with the group-II antagonist LY 341495 potentiated the release of glutamate. This potentiation of EAA release was not seen using the group-II and -III antagonist CPPG. CPPG is more selective for group-III mGluRs (Jane *et al.* 1996); therefore, it appears that antagonism of group-II but not group-III mGluRs potentiates the release of EAAs. Since mGluR3 may be more highly expressed in the spinal cord than mGluR2 (Ohishi *et al.* 1993, 1998; Berthele *et al.* 1999) and LY 341495 is more potent at mGluR3 (Kingston *et al.* 1998), it is likely that inhibition of mGluR3 mediates the potentiation of EAA release after treatment with LY 341495. In normal animals, group-II antagonists reduce thresholds to mechanical stimuli, suggesting that there may be tonic group-II activity in the spinal cord (Dolan and Nolan 2000; Neugebauer *et al.* 2000). Inhibiting this tonic activity prior to SCI may inhibit intracellular signaling

pathways that would normally reduce EAA release. However, it is unclear why antagonism of group-III mGluRs did not affect extracellular EAA levels when treatment with the group-III agonist L-AP4 did reduce EAA levels. Since mGluR-mediated responses are tightly regulated by receptor phosphorylation (Peavy and Conn 1998; Saugstad *et al.* 1998; Macek *et al.* 1999; Schaffhauser *et al.* 2000), it is possible that the activation states of group-II and group-III mGluRs change such that group-III mGluRs play a greater role in modulating neurotransmitter release after injury.

GABA release following SCI

The current study is in agreement with a previous report of an increase in extracellular GABA concentrations immediately following contusion injury to the spinal cord (Liu and McAadoo 1993). The temporal increase in extracellular GABA concentrations parallels the rise in extracellular EAAs; both are maximally increased within the first 15 min following SCI. Most of the increase in extracellular GABA after SCI is probably due to the destruction of GABAergic neurons by the initial mechanical damage and release of GABA from un-injured neurons due to the excitation induced from the large increase in extracellular EAAs. If similar mechanisms are responsible for the increase in extracellular GABA levels and EAAs following SCI, then it would be expected that treatments with mGluR agents that affect extracellular EAAs would also affect GABA levels. This is in fact what is seen in the current study: agents that decreased extracellular EAA levels (AIDA and LY 367385 + MPEP) produced the greatest decreases in extracellular GABA concentrations. Additional support comes from the results of treatment with LY 341495 and CPPG. Treatment with LY 341495 resulted in an increase in EAA levels at 30 min following SCI. This rise in EAA was accompanied by an increase in GABA levels, whereas treatment with CPPG did not affect extracellular EAA or GABA levels. Presynaptic mGluR2/3s are associated with GABA terminals in other regions of the CNS and may modulate GABA release from synaptic terminals (Hayashi *et al.* 1993; Ohishi *et al.* 1993, 1994; Stefani *et al.* 1994; Neki *et al.* 1996; Petralia *et al.* 1996). In the spinal cord, mGluR2/3 colocalizes with GABA, although there appears to be segregation between mGluRs and GABA immunopositive perikarya (Jia *et al.* 1999). This modest level of colocalization may explain the relative lack of effect on extracellular GABA levels using the group-II and -III agents in the current study.

Relationship between effects on EAA release and neuroprotection

Recently it was reported that treatment with LY 367385 confers neuroprotection following SCI (Mills *et al.* 2001b). Since LY 367385 did not affect extracellular EAA release in the current study, it seems unlikely that the antagonist-induced neuroprotective effects of mGluR1 are a result of a

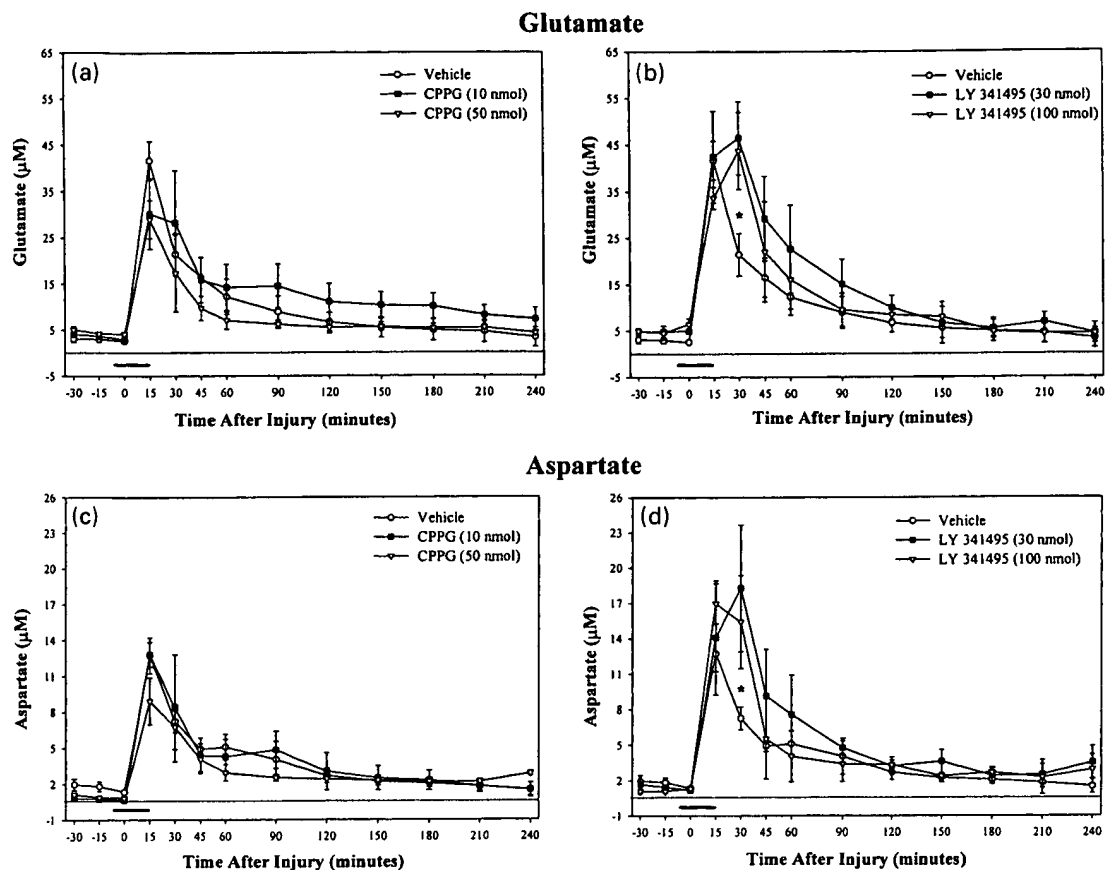


Fig. 7 Time course of extracellular glutamate (a and b) and aspartate (c and d) concentrations after SCI in CPPG- (a and c) and LY 341495-treated (b and d) groups. LY 341495, but not CPPG, increased extracellular EAAs at 30 min following injury. Data are

means \pm SEM. *Indicates a statistically significant difference between the vehicle and treatment groups ($p < 0.05$). Treatment duration is indicated by a horizontal line above the x-axis.

reduction in EAA release following injury. Furthermore, treatment with MPEP at doses that reduce EAA release following SCI does not result in overall tissue sparing (Mills *et al.* 2001b). Since the neuroprotective effects of both mGluR1 and mGluR5 appear to be independent of their effects on EAA release, it may be that inhibition of group-I mGluR-mediated intracellular pathways that lead to cell death (e.g. release of Ca^{2+} from intracellular stores) are responsible for the observed neuroprotective effects following SCI. Similarly, the dose of L-AP4 that reduces extracellular EAA levels in the current study did not produce significant neuroprotection following SCI in a previous study (Mills *et al.* 2001c). Previous reports using *in vitro* models of neuronal trauma indicate that at least some of the neuroprotective effects induced by antagonism of group-I mGluRs (Mukhin *et al.* 1996, 1997) and group-III mGluRs (Faden *et al.* 1997) are independent from mGluR

and NMDA receptor interactions. Taken together, these results suggest that modulation of extracellular glutamate concentrations may not be the primary mechanism by which mGluRs influence cell loss after CNS injury. Future experiments exploring this relationship and examining other mechanisms of mGluR mediated cell death following SCI are warranted.

Summary

The current study shows that group-I mGluRs modulate extracellular EAA and GABA levels following SCI. Antagonism of mGluR5 produced a greater reduction in extracellular EAAs than did antagonism to mGluR1 following SCI. When both treatments were combined, a greater reduction in extracellular EAAs was seen than for either individual treatment. Activation of group-III, but not

group-II, mGluRs following SCI reduced extracellular EAA levels without affecting GABA concentrations. Extracellular GABA concentrations followed a similar temporal pattern as the extracellular EAA concentrations. That is, both GABA and EAA levels were maximally increased during the first 15 min of injury. Previous reports show that group-I mGluR antagonists, but not group-II or -III agonists, are neuroprotective following SCI. These neuroprotective effects do not correlate with the effects of these agents on EAA release, suggesting that mGluR-mediated intracellular pathways not directly responsible for modulating neurotransmitter release may help determine cell survivability following CNS injury.

Acknowledgements

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Group II Metabotropic Glutamate Receptors Modulate Extracellular Glutamate in the Nucleus Accumbens

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ABSTRACT

The regulation of extracellular glutamate in the nucleus accumbens by group II metabotropic glutamate receptors (mGluR2/3) was examined in vivo. Stimulation of mGluR2/3 with 2*R*,4*R*-4-aminopyrrolidine-2,4-dicarboxylate (APDC) or *N*-acetylaspartyl-glutamate reduced extracellular glutamate levels. Conversely, blockade of mGluR2/3 by LY143495 or (*RS*)-1-amino-5-phosphonoindan-1-carboxylic acid (APICA) increased extracellular glutamate, an effect antagonized by the coadministration of APDC. These effects likely involve both vesicular and nonvesicular glutamate, because the increase in glutamate by APICA or the decrease by APDC was prevented by blocking N-type calcium channels and the release of glutamate after potassium-induced membrane depolarization was antagonized by APDC. In addition, blockade of the cystine-glutamate exchange, a major non-vesicular source of extracellular glutamate, by (*S*)-4-carboxy-

phenylglycine blocked the effects induced by either APDC or APICA. However, blockade of Na⁺ channels by tetrodotoxin or Na⁺-dependent glutamate transporters by *DL*-threo- β -benzyloxyaspartate failed to affect the alterations in extracellular glutamate by APICA or APDC, respectively. Group II mGluRs are G_i-coupled and coexpression with the cAMP-dependent protein kinase (PKA) activator Sp-cAMPS blocked the reduction in glutamate by APDC and the PKA inhibitor Rp-cAMPS prevented the elevation in glutamate by APICA. Taken together, these data support three conclusions: 1) group II mGluRs regulate both vesicular and nonvesicular release of glutamate in the nucleus accumbens, 2) there is tonic in vivo stimulation of mGluR2/3 by endogenous glutamate, and 3) modulation of group II mGluRs of extracellular glutamate is Ca²⁺- and PKA-dependent.

Metabotropic glutamate receptors (mGluRs) belong to a class of G protein-coupled receptors that is comprised of eight different subtypes that have been organized into three groups based upon sequence homology and coupling to intracellular messengers. Group I receptors (mGluR1,5) are coupled to phospholipase C, whereas group II (mGluR2,3) and group III (mGluR4,6,7,8) receptors are negatively coupled to adenylate cyclase (for review, see Conn and Pin, 1997). Group II and III mGluRs act to inhibit neurotransmitter release both as autoreceptors located on glutamatergic terminals or as presynaptic heteroreceptors. Extensive studies have emerged indicating that mGluRs play an important role in neuroplasticity (Anwyl, 1999), and various drugs targeting group II mGluRs have therapeutic potential including, protection from excitotoxicity, treatment of anxiety, Parkinson's disease, schizophrenia, and drug addiction (for review, see Conn and Pin, 1997). A possible role in addiction is indicated

by the recently described involvement of glutamate transmission in the nucleus accumbens (NAcc) and the possibility that reducing glutamate transmission by group II mGluR agonists may be of therapeutic benefit (Cornish and Kalivas, 2000; Vanderschuren and Kalivas, 2000).

Group II mGluRs are expressed in the nucleus accumbens (Ohishi et al., 1993a, 1993b; Testa et al., 1998). Selective activation of the group II mGluRs in the NAcc blocks amphetamine-induced locomotor behavior (Kim et al., 2000). In vitro electrophysiological studies in brain slices confirm that group II mGluRs inhibit glutamate release in the NAcc (Manzoni et al., 1997). Moreover, in vivo microdialysis studies show that group II agonists reduce extracellular dopamine in the NAcc (Hu et al., 1999).

Although the presence of group II mGluRs in the NAcc has been established, the identity and the properties of group II mGluRs in modulation of glutamate release remains unclear. For example, the basal level of extracellular glutamate is derived from both vesicular and nonvesicular sources (Timmerman and Westerink, 1997), and it is not known which

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ABBREVIATIONS: mGluRs, metabotropic glutamate receptors; APDC, (2*R*,4*R*)-4-aminopyrrolidine-2,4-dicarboxylate; NAAG, *N*-acetylaspartyl-glutamate; 2-PMPA, 2-(phosphonomethyl) pentanedioic acid; APICA, (*RS*)-amino-5-phosphonoindan-1-carboxylic acid; (*S*)-4CPG, (*S*)-4-carboxy-phenylglycine; TTX, tetrodotoxin; TBOA, *DL*-threo- β -benzyloxyaspartate; Sp-/Rp-cAMPS, Sp-/Rp-adenosine 3'5'-cyclic monophosphothioate triethylamine; PKA, cAMP-dependent protein kinase; ANOVA, analysis of variance; PLSD, protected least significant difference; NAcc, nucleus accumbens; NMDA, *N*-methyl-D-aspartate.

glutamate pool is modulated by group II mGluRs. In addition, activation of group II mGluRs has been shown to inhibit cAMP formation in *in vitro* expression systems, brain slices, and neuronal cultures, but it is unknown whether cAMP signaling is also mediating the effects of group II mGluRs *in vivo* (for review, see Conn and Pin, 1997). Thus, the present study used *in vivo* microdialysis combined with mGluR2/3 immunoblotting to characterize the modulation of extracellular glutamate by direct perfusion of various group II selective agonists or antagonists into the NAcc. Experiments were also conducted to examine the involvement of various ion channels, the cystine-glutamate exchanger, glutamate transporters, and the intracellular cAMP/c-AMP-dependent protein kinase (PKA) signaling cascade in mGluR modulation of glutamate release.

Materials and Methods

Animals Housing and Surgery. All experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The subjects were male Sprague-Dawley rats (Charles Rivers Laboratories, Inc., Wilmington, MA) weighing 250 to 275 g upon arrival and were individually housed in an American Association for Laboratory Animal Care-approved facility maintained on a 12-h light/dark cycle (lights on 7 AM). All experimentation was conducted during the light period. Using ketamine (100 mg/kg) and xylazine (3 mg/kg) anesthesia, dialysis guide cannulae (20 gauge, 14 mm; Small Parts, Roanoke, VA) were implanted over the nucleus accumbens [+1.6 mm anterior to Bregma, \pm 1.6 mm mediolateral, -4.7 mm ventral to the skull surface according to the atlas of Paxinos and Watson (1986)] using a 6° angle from vertical. The guide cannulae were fixed to the skull with four stainless steel skull screws (Small Parts) and dental acrylic. Surgeries were performed 5 to 7 days after arrival of the subjects, and dialysis experiments were begun 1 week after the surgical procedure.

In Vivo Microdialysis. The night before the experiment, concentric microdialysis probes (with 2 mm of active membrane) were inserted 3 mm beyond tips of guide cannulae into the nucleus accumbens. Dialysis buffer (5 mM KCl, 140 mM NaCl, 1.4 mM CaCl₂, 1.2 mM MgCl₂, 5.0 mM glucose, plus 0.2 mM phosphate-buffered saline to give a pH of 7.4) was advanced through the probe at a rate of 2 μ l/min via syringe pump (Bioanalytical Systems, West Lafayette, IN). Beginning at 2 h after turning on the pump at 8 AM the next morning, baseline samples were collected at 20-min intervals for 100 min. After collecting the baseline samples various drugs were administered via reverse dialysis into the NAcc.

Multiple doses of each mGluR agonist or antagonist were administered alone or in combination with other drugs. Dosage ranges of the various drugs were based upon the relative EC₅₀ or IC₅₀ values for binding to the respective receptors. *N*-acetylaspartylglutamate (NAAG) was purchased from Sigma-RBI (Natick, MA), and all other mGluR compounds, including (2*R*,4*R*)-aminopyrrolidine-2,4-dicarboxylate (APDC), (1*R*)-1-amino-5-phosphonindan-1-carboxylic acid (APICA), LY143495, and (S)-4-carboxyphenylglycine [(S)-4CPG] were purchased from Tocris (Ballwin, MO). NAAG was dissolved with filtered dialysis buffer (see below), whereas all other mGluR compounds were initially dissolved in 0.1 N NaOH (Sigma, St. Louis, MO) and neutralized with 0.1 N HCl (Sigma) to a concentration of 10⁻² M. Working concentrations were then made by diluting with filtered dialysis buffer. Diltiazem and tetrodotoxin (TTX) were purchased from Tocris, and ω -conotoxin GVIA, Sp- and Rp-adenosine 3',5'-cyclic monophosphothioate triethylamine (Sp-cAMPS, Rp-cAMPS) were obtained from Sigma-RBI. 2-(Phosphonomethyl) pentanedioic acid (2-PMPA) was a gift from Guilford Pharmaceuticals, Inc. (Baltimore, MD)

and DL-threo- β -benzyloxyaspartate (TBOA) was a gift from Dr. Keiko Shimamoto (Suntory Institute for Bioorganic Research, Osaka, Japan). All of the drugs were dissolved with filtered dialysis buffer and were freshly prepared on day of the experiment. In some experiments KCl was used to increase glutamate release and in these experiments NaCl was reduced proportionally to retain iso-osmolality.

Quantification of Glutamate. The concentration of glutamate in the dialysis samples was determined using HPLC with fluorimetric detection. The dialysis samples were collected into 10 μ l of 0.05 M HCl containing 2 pmol of homoserine as an internal standard. The mobile phase consisted of 13% acetonitrile (v/v), 100 mM Na₂HPO₄, and 0.1 mM EDTA, pH 6.04. A reversed-phase column (10 cm, 3 μ m ODS; Bioanalytical Systems, West Lafayette, IN) was used to separate the amino acids, and precolumn derivatization of amino acids with *o*-phthalaldehyde was performed using a model 540 autosampler (ESA, Inc., Chelmsford, MA). Glutamate was detected by a fluorescence spectrophotometer (Linear Fluor LC 305; ESA Inc.) using an excitation wavelength of 336 nm and an emission wavelength of 420 nm. The area under curve of the glutamate and homoserine peaks was measured with ESA 501 Chromatography Data System. Glutamate values were normalized to the internal standard homoserine and compared with an external standard curve for quantification. The limit of detection for glutamate was 1 to 2 pmol.

mGluR2/3 Immunoblotting. To determine the existence of mGluR2/3 proteins in the NAcc, eight rats were decapitated, and the brains were rapidly removed and dissected into coronal sections on ice. The appropriate brain regions were sampled on an ice-cooled Plexiglas plate using a 15-gauge tissue punch, including the prefrontal cortex, parietal cortex, ventral tegmental area, dorsolateral striatum, medial nucleus accumbens (predominately medial shell), and lateral nucleus accumbens (core). Brains punches were immediately frozen on dry ice and stored at -80°C until homogenized for immunoblotting.

The dissected brain punches were homogenized with a hand-held tissue grinder in homogenization medium (0.32 M sucrose, 2 mM EDTA, 1% sodium dodecyl sulfate, 50 μ M phenyl methyl sulfonyl fluoride, and 1 μ g/ml leupeptin, pH 7.2), subjected to low-speed centrifugation (2000g, to remove insoluble material) and stored at -80°C. Protein determinations were performed using the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Samples (30 μ g) were subjected to sodium dodecyl sulfate-polyacrylamide gel (8%) electrophoresis utilizing a mini-gel apparatus (Bio-Rad), transferred via semidry apparatus (Bio-Rad) to nitrocellulose membrane, and probed for the proteins of interest (1 gel/protein/brain region). mGluR2/3 was identified using a rabbit anti-rat antibody (1:3000) purchased from Upstate Biotech (Lake Placid, NY) that was made against a peptide containing the C terminus. In control experiments a synthesized peptide having the same 21 amino acid sequence on the C terminus of mGluR2/3 was used to competitively inhibit the binding of antibody to mGluR2/3. Labeled proteins were detected using an horseradish peroxidase-conjugated anti-rabbit secondary IgG diluted 1:30,000 (Upstate Biotech) and visualized with enhanced chemiluminescence (Amersham Life Sciences, Arlington Heights, IL). Assurance of even transfer of protein was evaluated with Ponceau S (Sigma) followed by destaining with de-ionized water. Immunoreactive levels were quantified by integrating band density \times area using computer-assisted densitometry (NIH Image version 1.60). The density \times area measurements were averaged over three control samples for each gel and all bands were normalized as percent of the control values.

Histology. After the dialysis experiments, rats were administered an overdose of pentobarbital (>100 mg/kg i.p.) and transcardially perfused with 0.9% saline followed by 10% formalin solution. Brains were removed and placed in 10% formalin for at least 1 week to ensure proper fixation. The tissue was blocked, and coronal sec-

tions (100 μm thick) were made through the site of dialysis probe with a vibratome. The brains were then stained with cresyl violet to verify anatomical placement according to the atlas of Paxinos and Watson (1986).

Statistical Analysis. The StatView statistics package was used to estimate statistical significance. A one-way ANOVA with repeated measures over dose was used to determine the effect of individual drugs on extracellular glutamate levels. A two-way ANOVA with repeated measures over time or dose were used to compare between treatments. Upon identification of statistical significance, post hoc comparisons were made with a Fischer's PLSD.

Results

mGluR2/3 Immunoproteins Are Highly Expressed in the Nucleus Accumbens. A high density of mGluR2/3 immunoproteins were detected in many brain regions including the shell and core of the nucleus accumbens, prefrontal cortex, ventral tegmental area, and striatum of rats. Both dimer and monomer forms were detected, and the dimer was the predominant form of mGluR2/3 in all brain nuclei examined. Figure 1 shows representative immunoblots that illustrate the two forms of mGluR2/3 proteins in the nucleus accumbens (shell and core) and prefrontal cortex. Figure 1 also shows that both the dimer and monomer forms could be completely absorbed by a synthetic peptide having the identical 21 amino acid sequence with the C terminus of mGluR2/3.

Group II mGluRs Reduce Extracellular Glutamate Levels in the Nucleus Accumbens. A selective agonist or antagonist for mGluR2/3 was perfused into the accumbens by reverse microdialysis and the levels of extracellular glutamate were estimated. Figure 2A shows that the mGluR2/3 agonist APDC elicited a dose-dependent decrease in extracellular glutamate levels and this effect was attenuated by the specific group II mGluR antagonist APICA (Fig. 2B). The threshold dose for producing a significant reduction was 5 μM APDC, and the reduction in extracellular glutamate was reversed by washing out the drug with dialysis buffer. Furthermore, NAAG, a mGluR3 agonist (Wroblewska et al., 1997; Schweitzer et al., 2000) elicited a dose-dependent decrease in extracellular glutamate levels in the nucleus accumbens (Fig. 2, C and D). The minimal effective dose of NAAG was 10 μM . The experiment was conducted in the presence of 500 μM 2-PMPA to inhibit the formation of glutamate derived from the metabolism of NAAG by NAALADase (Slusher et al., 1999). In the absence of 2-PMPA the capacity of NAAG to inhibit extracellular glutamate could not be demonstrated (data not shown). 2-PMPA

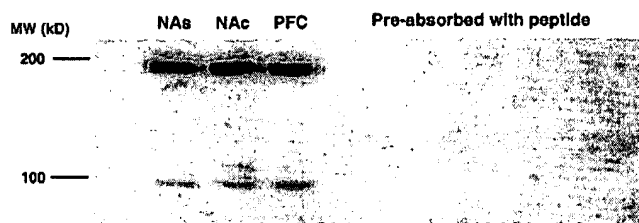


Fig. 1. mGluR2/3 immunoreactivity in the rat brain. The top panel shows representative Western blots demonstrating that two bands (left three lanes) were detected in the shell and core of the nucleus accumbens and the prefrontal cortex (PFC) corresponding to the monomer and dimer of mGluR2/3. Both bands were completely absorbed by a synthetic peptide having the identical 21 amino acids to the C-terminal of mGluR2/3 (right three lanes).

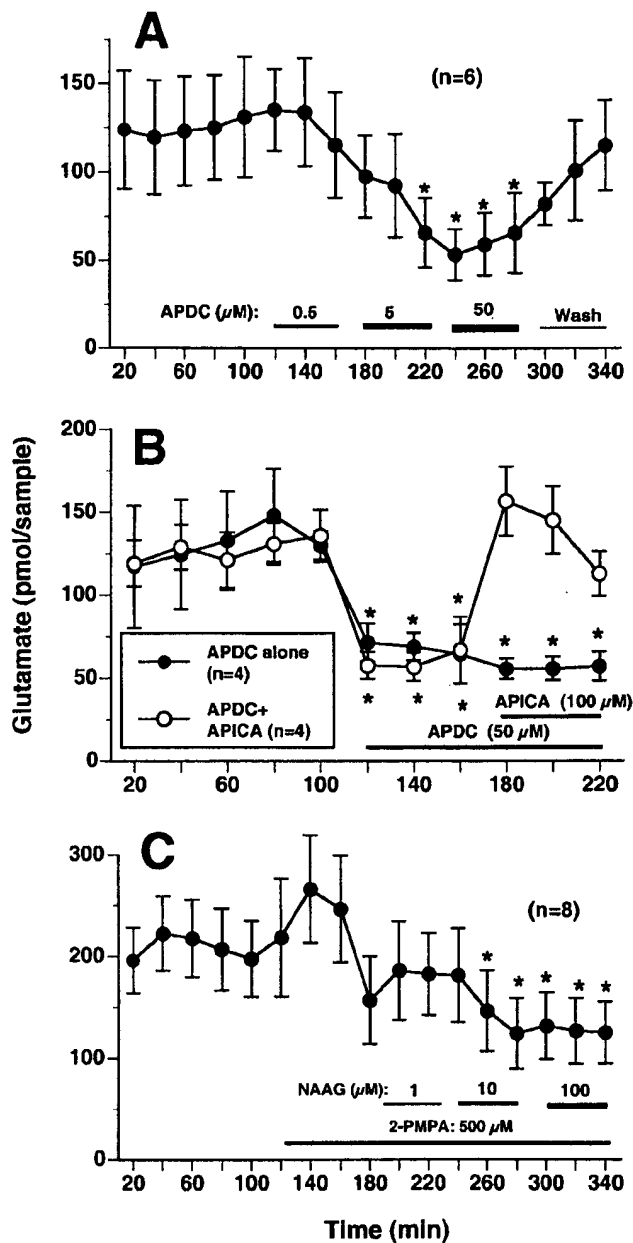


Fig. 2. Group II mGluR agonists decrease extracellular glutamate in the nucleus accumbens. A, reverse dialysis of the mGluR2/3 agonist APDC produced a reversible, dose-dependent decrease in extracellular glutamate levels. One-way ANOVA with repeated measurement over doses reveals that this decrease in glutamate by APDC is significant ($F_{(16,113)} = 6.54, p < 0.05$). Each point represents mean \pm S.E.M. of picomoles of glutamate per 20-min sample. B, the inhibitory effect of 50 μM APDC was competitively reversed by coadministration of 100 μM APICA, a selective group II antagonist. C, the selective mGluR3 agonist NAAG dose dependently decreased extracellular glutamate in the nucleus accumbens (one-way ANOVA over doses with repeated measurement; $F_{(22,132)} = 54.12, p < 0.001$) in the presence of 500 μM 2-PMPA, to inhibit NAAG degradation by NAALADase. *, $p < 0.05$, compared with the average of the last three of the five baseline samples using a Fisher PLSD for post hoc comparisons. $n = XX$ on each figure indicates animal number in each group of study.

alone did not significantly alter the extracellular levels of glutamate (Fig. 2C).

Conversely, perfusion of the mGluR2/3 antagonist APICA or LY143495 into the nucleus accumbens produced a dose-

dependent increase in extracellular glutamate (Fig. 3). Further the increase by APICA was reversed by coprefusion of the group II agonist APDC (Fig. 3B). The threshold dose for a significant response by APICA and LY143495 was 10 μ M and 10 nM, respectively, and drug washout with dialysis buffer reversed the increase by the highest dose of APICA (1 mM).

mGluR2/3 Modulation of Extracellular Glutamate is Ca^{2+} -Dependent. Basal extracellular glutamate derives from both neuronal and glial sources, and can be derived from vesicular or cytoplasmic pools (Timmerman and Wes-

terink, 1997). Vesicular neurotransmitter release by high K^+ is predominantly Ca^{2+} -dependent (for review, see Timmerman and Westerink, 1997). To determine whether the reduction in extracellular glutamate by the group II mGluR agonists is derived from vesicular stores of glutamate, the capacity of APDC to reverse the release of glutamate by a high concentration of K^+ (80 mM) was examined. Figure 4, A and B, illustrate that the high K^+ -evoked glutamate release was significantly inhibited by the coadministration of 50 μ M APDC. In further support of a role for Ca^{2+} -dependent vesicular release of glutamate, either the L- or N-type Ca^{2+} channel blockers diltiazem or ω -conotoxin GVIA, respectively, was coinfused into the NAcc with APICA. Either drug completely blocked the elevation of extracellular glutamate produced by APICA (Fig. 5A). Whereas diltiazem alone had no significant effect, ω -conotoxin GVIA alone significantly reduced the basal level of extracellular glutamate by 30 to 40%. Furthermore, coadministration of ω -conotoxin GVIA blocked the capacity of APDC to reduce extracellular glutamate (Fig. 4B). These data suggest that the reduction in basal extracellular glutamate by N-type Ca^{2+} channel blockade and mGluR2/3 stimulation were not additive and may involve the same or overlapping mechanisms.

In contrast to the involvement of extracellular Ca^{2+} , pretreatment with the voltage-dependent Na^+ channel blocker TTX at a dose sufficient to nearly eliminate detectable extracellular levels of monoamine transmitters (1 μ M; Timmerman and Westerink, 1997) did not block the dose-dependent increase in extracellular glutamate elicited by APICA (Fig. 5C). TTX alone did not significantly alter the basal concentration of glutamate. This result argues that mGluR2/3 directly regulates Ca^{2+} -dependent release of glutamate and is not acting indirectly via a *trans*-synaptic mechanism. Although Na^+ -dependent glutamate transporters play an important role in modulating the basal level of extracellular glutamate, blockade of glutamate uptake by TBOA, a broad-spectrum glutamate uptake inhibitor (Shimamoto et al., 1998), did not attenuate the APDC-induced reduction in glutamate (Fig. 5D), suggesting that the effect of mGluR2/3 stimulation on extracellular glutamate is independent of glutamate transporters.

mGluR2/3 Involves Cystine-Glutamate Exchange. The basal level of extracellular glutamate measured by microdialysis is predominantly controlled by cystine-glutamate exchange, which provides the primary source of extracellular, nonvesicular glutamate (Baker et al., 2001). To determine whether group II mGluRs might reduce extracellular glutamate by negatively modulating cystine/glutamate exchange, the inhibitor of cystine/glutamate exchange (S)-4CPG (Ye et al., 1999) was infused into the NAcc. Coinfusion of (S)-4CPG with APICA or APDC prevented the increase in glutamate by APICA or the decrease by APDC (Fig. 6, A and B). (S)-4CPG (5 μ M) alone decreased extracellular glutamate by approximately 50% (Fig. 6A).

Signaling through PKA Mediates Group II mGluR Reduction in Extracellular Glutamate. Group II mGluRs are negatively coupled to adenylate cyclase and PKA via inhibitory G_i proteins (Conn and Pin, 1997; Anwyl, 1999). To evaluate a role for PKA in the capacity of mGluR2/3 to modulate extracellular glutamate levels, the PKA activator Sp-cAMPS or the PKA inhibitor Rp-cAMPS

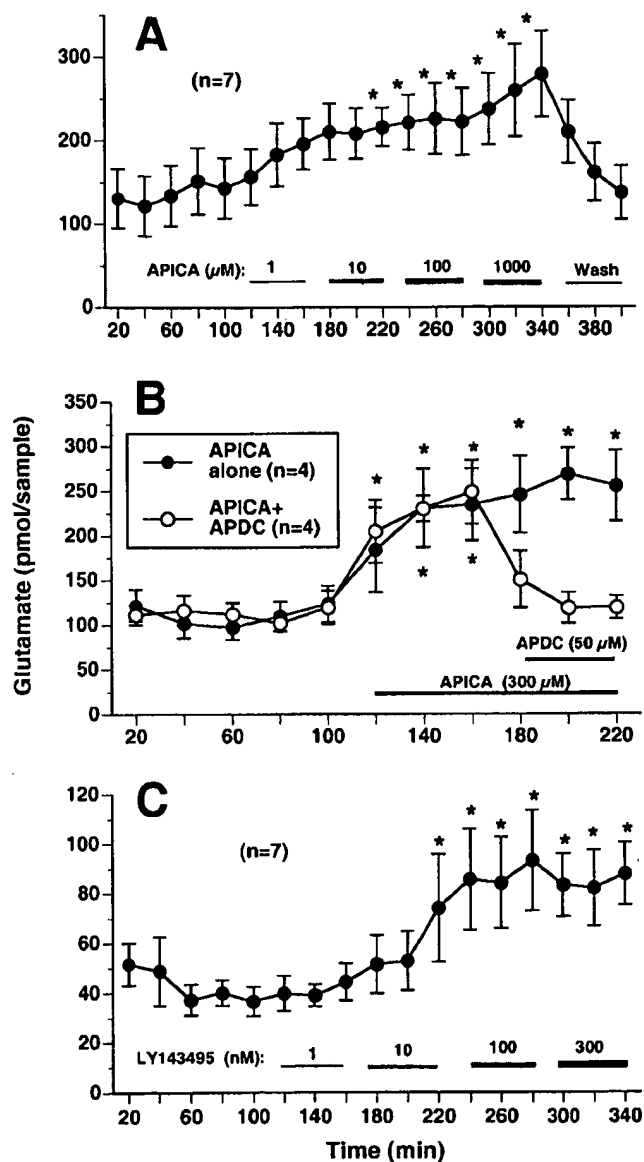


Fig. 3. Group II mGluR antagonists elevate extracellular glutamate in the nucleus accumbens. A, administration of the group II mGluR antagonist APICA dose dependently increased extracellular glutamate levels over the entire dose range tested ($F_{(10,64)} = 6.87$, $p < 0.001$). B, the increase in extracellular glutamate by 300 μ M APICA was blocked by coadministration of 50 μ M APDC. C, LY143495, another highly potent, selective group II antagonist, dose dependently increased extracellular glutamate ($F_{(10,64)} = 9.74$, $p < 0.05$). *, $p < 0.05$, compared with the average of the last three of the five baseline samples using a Fisher PLSD for post hoc comparisons.

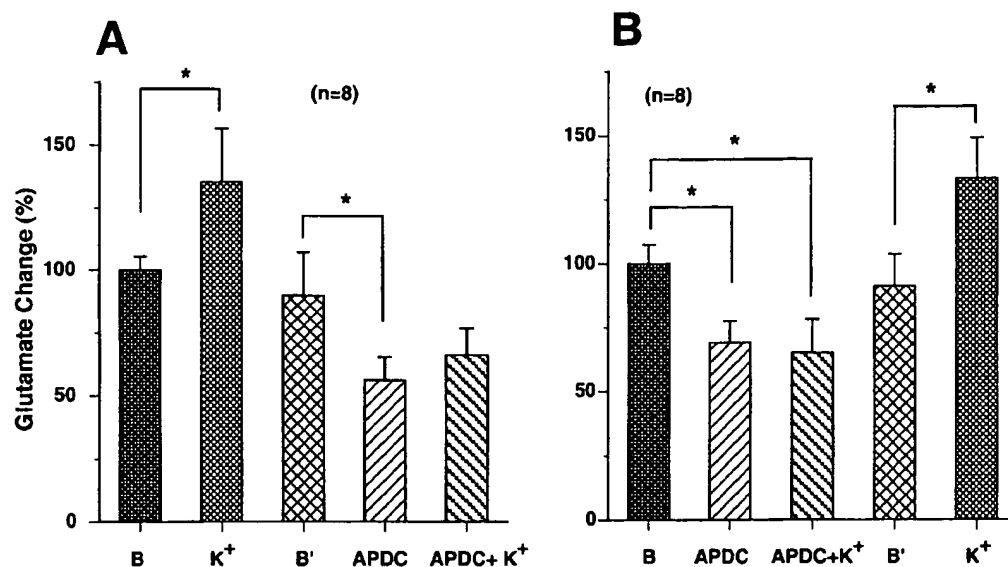


Fig. 4. The group II mGluR agonist 50 μ M APDC blocked 80 mM K⁺-stimulated glutamate release in the nucleus accumbens. Each panel is a separate experiment giving the same drug treatment in a different sequence to verify that the drug would wash out and not produce enduring impairment of K⁺-evoked glutamate release. A one-way ANOVA with repeated measures over time (each drug treatment) indicated that K⁺ stimulation and APDC significantly altered the basal level of extracellular glutamate (A, $F_{(4,39)} = 13.31$, $p < 0.001$; B, $F_{(4,39)} = 4.23$, $p < 0.005$). *, $p < 0.05$, compared with the average of each baseline (B or B') before the drug administration.

was perfused into the accumbens via the dialysis probe in combination with the mGluR2/3 agonist APDC or the antagonist APICA. Figure 7, A and C show the effect of increasing doses of Sp-cAMPS or Rp-cAMPS alone. Although Sp-cAMPS elevated glutamate levels at lower doses and decreased levels at higher doses, Rp-cAMPS reduced glutamate at lower doses and increased levels at higher doses. Based upon these dose-response curves a relatively low dose of each drug (5 nM) was coadministered with APDC or APICA. Figure 7B shows that Sp-cAMPS attenuated the APDC-induced decrease in extracellular glutamate. Conversely, Rp-cAMPS inhibited APICA-induced increase in extracellular glutamate (Fig. 7D). The inhibitory effect of both Sp-cAMPS and Rp-cAMPS were reversible because after wash-out with dialysis buffer, the capacity of APDC to reduce and APICA to elevate extracellular glutamate was restored.

Histology. Figure 8 depicts the dialysis probe placements in the nucleus accumbens. The majority of probe placements in the nucleus accumbens were at or medial to the anterior commissure. Placements tended to be primarily in the core of the nucleus accumbens, although a number were located at the interface between the core and either the medial or the ventral limb of the shell, and a minority of placements were primarily in the shell. In addition, some probes were partly (<30%) dorsal to the nucleus accumbens in the striatum or septal region.

Discussion

These data provide *in vivo* evidence that pharmacological stimulation of group II mGluRs in the nucleus accumbens reduces the basal concentration and K⁺-evoked increases in extracellular glutamate. Moreover, group II mGluRs bear significant endogenous tone because blockade of mGluR2/3 elevates extracellular glutamate levels. The effects of the group II compounds were shown to require active L- and N-type Ca²⁺ conductances, as well as functional cystine-glutamate exchange, and to be signaled through cAMP/PKA cascade. In contrast, there was no role

identified for voltage-dependent sodium channels or glutamate transporters.

Group II mGluRs Act as Autoreceptors to Inhibit Presynaptic Glutamate Release in the Nucleus Accumbens. *In vitro* electrophysiological experiments have revealed that a prominent physiological effect of mGluR2/3 agonists in the cortex and hippocampus is to reduce glutamatergic transmission by stimulating presynaptic autoreceptors (Anwyl, 1999), which has been confirmed as well in studies examining *in vitro* glutamate release (Cartmell and Schoepp, 2000). Although two *in vivo* studies have revealed the capacity of systemically administered mGluR2/3 agonist to reduce evoked glutamate release in the prefrontal cortex and striatum (Battaglia et al., 1997; Moghaddam and Adams, 1998), the present study is the first *in vivo* demonstration that locally stimulating group II mGluRs lowers extracellular glutamate. Moreover, the *in vivo* measurements revealed the presence of substantial tone by endogenous glutamate on mGluR2/3 in the nucleus accumbens. Thus, blocking mGluR2/3 elevated extracellular glutamate, and consistent with an action on presynaptic glutamate terminals, the increase was blocked by L- and N-type Ca²⁺ channel antagonists, but not by blocking voltage-dependent Na⁺ channels. Although N- and P/Q-types of Ca²⁺ channels are thought to mediate vesicular glutamate release from nerve terminals (Anwyl, 1991, 1999), L channels are predominantly located on soma, dendrites, and/or glial cells (Anwyl, 1999; Nachman-Clewner et al., 1999). Diltiazem blocked the APICA-induced increase in glutamate, suggesting that the L-type Ca²⁺ channels and mGluR2/3 on somatodendrites or glial cells, rather than just presynaptic mGluR2/3, are playing a role in modulating vesicular and/or nonvesicular glutamate release. Also consistent with a presynaptic site of action is the inhibition of the K⁺-mediated release of glutamate by APDC.

To further determine the involvement of the subtypes of group II mGluRs, the effect of the mGluR3 agonist NAAG (Wroblewska et al., 1997; Schweitzer et al., 2000) was examined. NAAG decreased extracellular glutamate levels in the

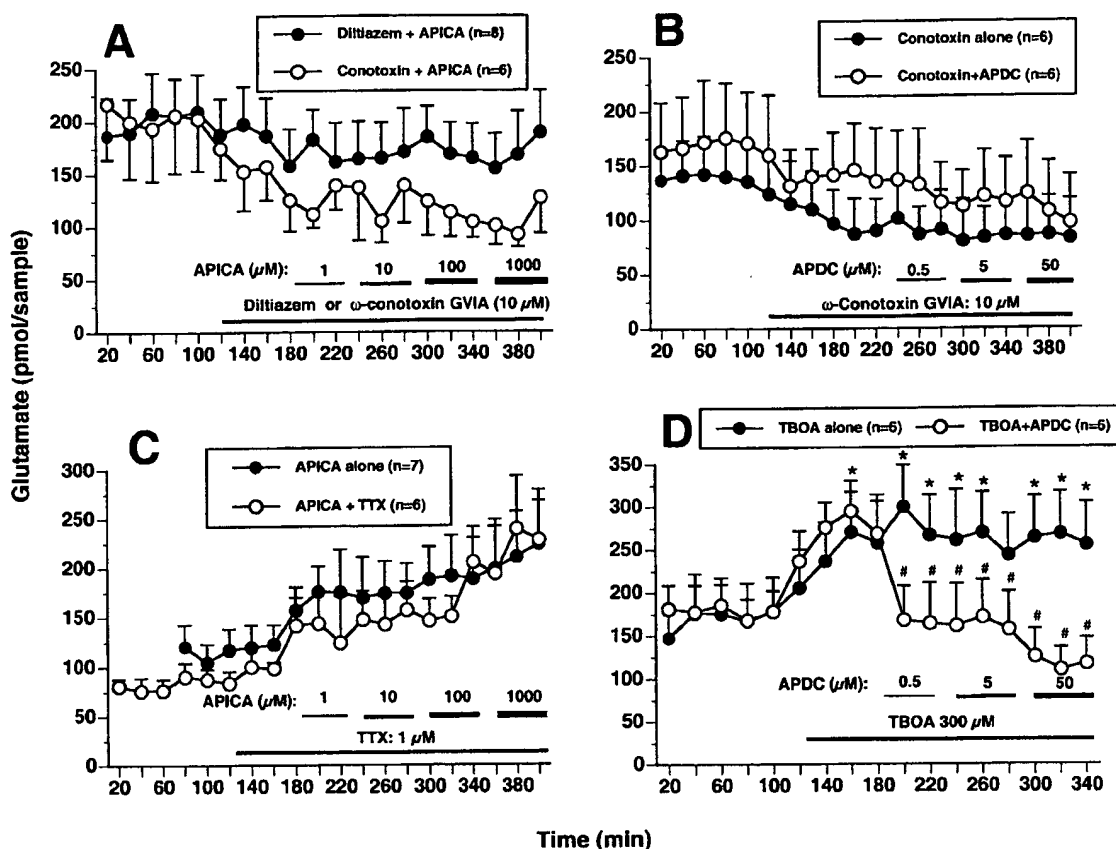


Fig. 5. Involvement of calcium channels, sodium channels, or glutamate transporters in mGluR2/3 regulation of extracellular glutamate. **A**, coadministration of L- and N-type Ca^{2+} channel blockers diltiazem (10 μM) and ω -conotoxin GVIA (10 μM), respectively, blocked APICA-induced increase in extracellular glutamate. After collecting five baseline samples the Ca^{2+} channel antagonists were introduced into the dialysis buffer for the remainder of the experiment as indicated by the bars. A one-way ANOVA with repeated measurement over the entire dose range indicates that APICA did not increase extracellular glutamate in the presence of either diltiazem or ω -conotoxin GVIA. **B**, N-type Ca^{2+} channel blocker ω -conotoxin GVIA antagonized the APDC-induced decrease in glutamate. A two-way ANOVA with repeated measures over time indicates that the extracellular glutamate level was significantly decreased over time (drug treatment) ($F_{(19,190)} = 4.5$, $p < 0.001$), but no significant treatment \times time interaction ($F_{(19,190)} = .35$, $p > 0.05$) was observed. ω -Conotoxin GVIA alone decreased the basal level of extracellular glutamate by around 30 to 40% beginning at 60 min after introduction into the dialysis buffer ($F_{(19,119)} = 3.39$, $p < 0.001$). **C**, coprefusion of the Na^{+} -channel blocker tetrodotoxin (TTX, 1 μM) failed to block the APICA-induced increase in extracellular glutamate. A two-way ANOVA with repeated measures over doses (time) revealed a significant increase in extracellular glutamate ($F_{(12,120)} = 3.87$, $p < 0.05$), but no significant treatment \times time interaction ($F_{(12,120)} = .39$, $p > 0.05$). TTX alone had no significant effect on the basal level of extracellular glutamate. **D**, blockade of glutamate transporters with TBOA failed to alter the inhibitory effect of APDC in glutamate. A two-way ANOVA with repeated measures over time reveals a significant drug effect ($F_{(16,160)} = 4.1$, $p < 0.001$) and treatment \times time interaction ($F_{(16,160)} = 5.13$, $p < 0.001$). *, $p < 0.05$, compared with the average of the last three baseline samples. #, $p < 0.05$, comparing TBOA+APDC with TBOA alone at each collection time.

presence of 2-PMMA, an enzyme (NAALADase) inhibitor that prevented NAAG metabolism to glutamate (Slusher et al., 1999). These data indicate that the mGluR3 contributes to the decrease in glutamate by APDC. However, no selective mGluR3 antagonist was available to further verify the role of mGluR3 in modulating endogenous glutamate release. Also, it was reported that NAAG is only 10-fold more selective for mGluR3 than mGluR2 (Cartmell et al., 1998; Schweitzer et al., 2000). Although NAAG may also acts as a weak NMDA agonist, the inhibitory effect of NAAG on glutamate release is unlikely mediated by activating NMDA receptors because previous studies have shown that NMDA receptor activation increases glutamate release in the striatum (Hashimoto et al., 2000).

Group II mGluRs Decrease in Extracellular Glutamate May Involve Cystine-Glutamate Exchange. The cystine-glutamate exchanger is a major nonvesicular source of glutamate. This exchanger is driven by the rela-

tive intra- and extracellular substrate gradients and typically operates to transport glutamate out and cystine into the cell (Kato et al., 1993; Warr et al., 1999). The cystine-glutamate exchanger was recently cloned and is found in a variety of tissue types, indicating that it is a primary metabolic source of intracellular cystine. Elevation of extracellular cystine concentration increased glutamate release from brain slices (Warr et al., 1999), an effect that was blocked by the relatively selective cystine-glutamate exchanger inhibitor (S)-4CPG (Ye et al., 1999). More recently, Baker et al. (2001) used in vivo microdialysis to show that the basal, extracellular glutamate content is derived mainly from cystine-glutamate exchange, because blockade of the cystine-glutamate exchanger by homocysteic acid or (S)-4CPG lowered extracellular glutamate levels by 60 to 70%. In the present study, pretreatment with (S)-4CPG prevented the increase in basal extracellular glutamate by APICA or the decrease by APDC. The former action re-

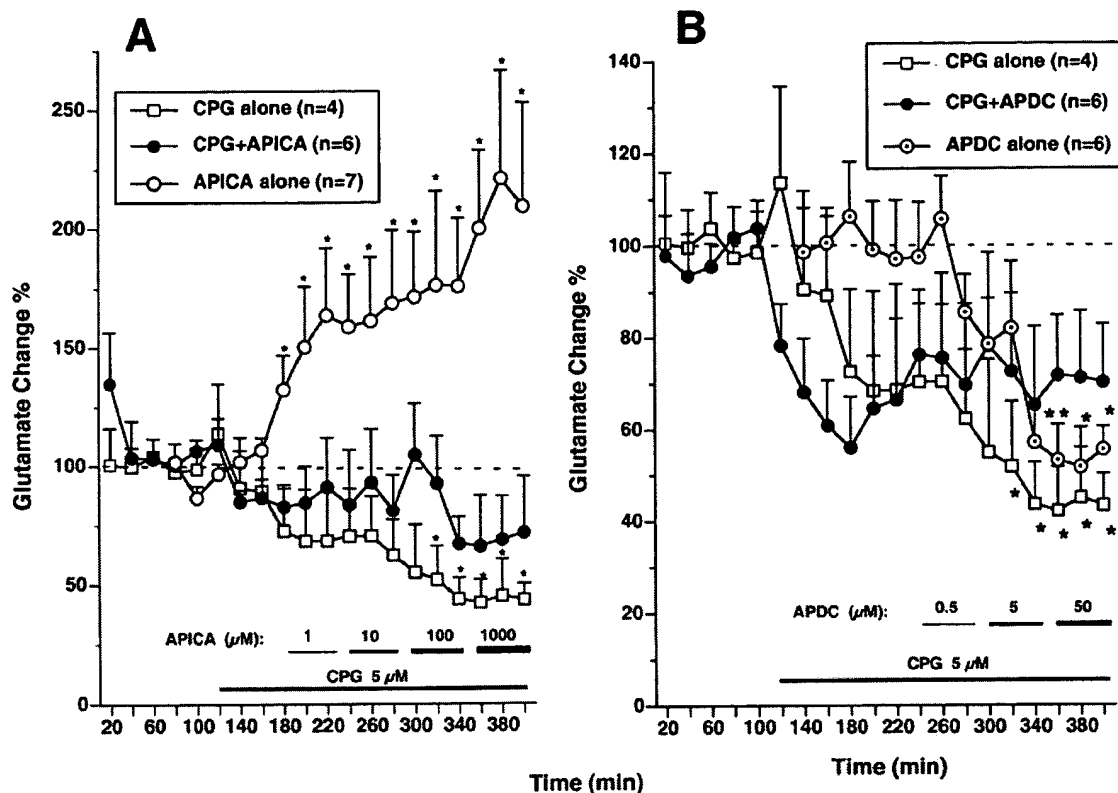


Fig. 6. Group II mGluRs inhibit cystine/glutamate exchanger. A, coadministration of the cystine/glutamate exchanger (S)-4CPG (5 μ M) blocked the APICA-induced increase in extracellular glutamate. APICA alone dose dependently increases ($F_{(2,17)} = 6.22, p < 0.05$), whereas 5 μ M (S)-4CPG alone significantly lowered extracellular glutamate levels ($F_{(5,17)} = 5.08, p < 0.05$). A one-way ANOVA with repeated measurement over the entire dose range indicates that APICA did not increase extracellular glutamate in the presence of (S)-4CPG. B, APDC dose dependently decreased extracellular glutamate ($F_{(5,13)} = 4.15, p < 0.05$), which was prevented in the presence of 5 μ M (S)-4CPG. A one-way ANOVA with repeated measures over time indicates that APDC did not decrease extracellular glutamate in the presence of the (S)-4CPG. *, $p < 0.05$, compared with the average of the last three baseline samples in each experimental group.

flects decreased glutamate tone by inhibiting cystine-glutamate exchange (Baker et al., 2001), while the latter suggests that cystine-glutamate exchange, at least in part, mediates the action of the group II mGluRs. Although (S)-4CPG acts as a group I mGluR antagonist, blockade of group I mGluRs does not alter extracellular glutamate (Baker et al., 2001). The mechanism by which mGluR2/3 may couple to the cystine-glutamate exchanger is unclear. However, the reversal of mGluR2/3 effects on extracellular glutamate by modulating PKA activity indicates that mGluR2/3 inhibition of PKA may be signaling changes in cystine-glutamate exchange.

PKA- and Calcium-Dependent Effects by Group II mGluRs. The most well-characterized signaling event for group II mGluRs is G_i -coupled reductions in cAMP formation and the subsequent inhibition of PKA (for review, see Conn and Pin, 1997). Electrophysiological studies demonstrate that activation of the adenylate cyclase cascade increases glutamatergic transmission in striatum and hippocampal slices, and may be critical in some forms of long-term potentiation (Colwell and Levine, 1995; Trudeau et al., 1996). Furthermore, Chavis et al. (1998) showed that activation of the cAMP/PKA cascade enhances presynaptic vesicle recycling at cerebellar granule cells. Consistent with a role for this signaling cascade in the present study, coapplication of the selective PKA activator Sp-

cAMPS blocked APDC-induced inhibition of glutamate release, whereas the selective PKA inhibitor Rp-cAMPS antagonized APICA-induced increase in extracellular glutamate.

Group II mGluRs have been previously shown to be G_i -coupled, negative modulators of L- and N-type Ca^{2+} channels in electrophysiological studies using brain slices, neuronal cultures, and heterologous expression systems (Chivas et al., 1994; Schumacher et al., 2000). Similarly, in vitro and in vivo release studies reveal L- and N-type channel involvement in the inhibition of dopamine release by group II mGluRs (Hu et al., 1999). In the present study, we observed that coadministration of the Ca^{2+} channel antagonists diltiazem (L-type) or ω -conotoxin GVIA (N-type) abolished the capacity of the group II antagonist to elevate or the agonist to reduce extracellular glutamate. Surprisingly, one electrophysiological investigation reported that blockade of N-type Ca^{2+} channels did not prevent the inhibition of the glutamate transmission induced by group II agonists in the nucleus accumbens (Manzoni et al., 1997). Possible reasons for the distinction from the present study include the use of the less selective mGluR2/3 agonist (2S,1'S,2'S)-2-(2'-carboxy-3',3'-difluorocyclopropyl)glycine and the fact that in the present study N-type channel blockade was maintained for 1 h before administering APDC, which may have resulted in a more complete blockade of the channels. Finally, phosphorylation of presynaptic proteins by

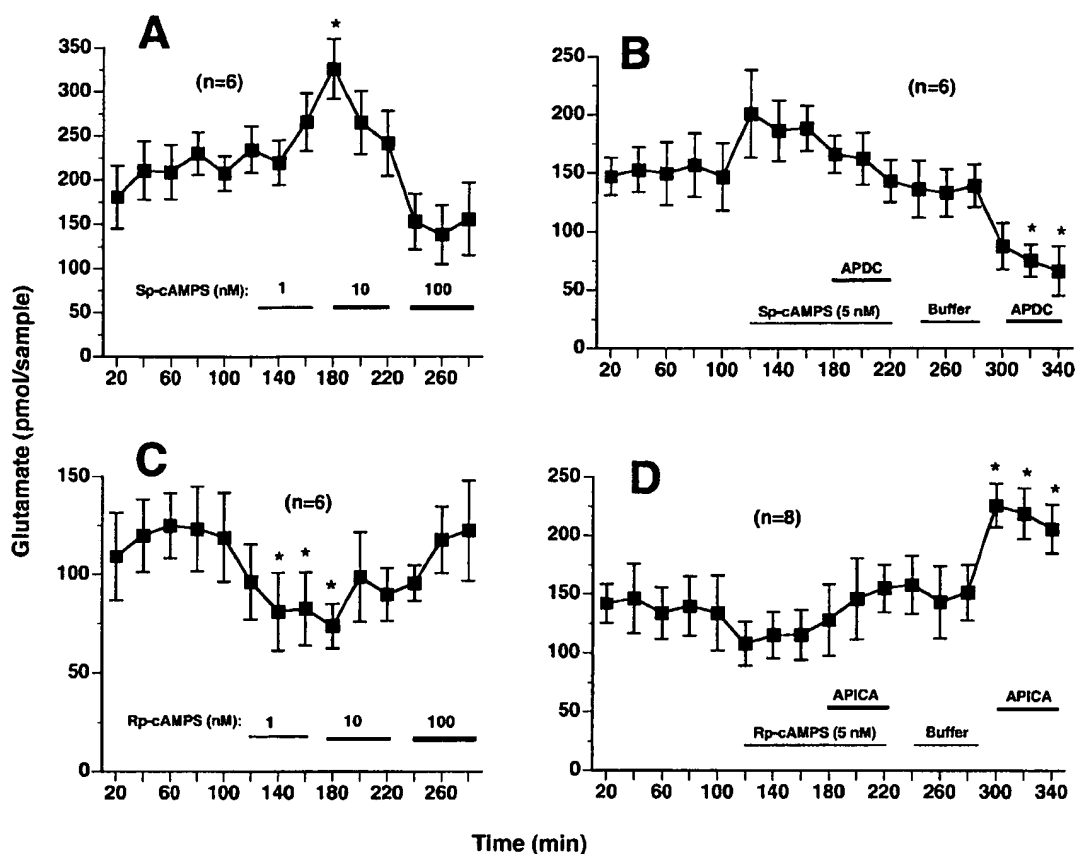


Fig. 7. Intracellular cAMP/PKA cascade mediates group II mGluR modulation of extracellular glutamate. **A**, dose-dependent effects of Sp-cAMPS, a cAMP-dependent protein kinase activator (one-way ANOVA with repeated measures over dose; $F_{(22,137)} = 3.37, p < 0.001$). Lower doses (1–10 nM) of Sp-cAMPS increased, whereas higher doses (10–1000 nM) decreased basal extracellular glutamate in the NAcc. **B**, coadministration of 5 nM Sp-cAMPS significantly reduced the capacity of APDC to decrease extracellular glutamate ($F_{(16,67)} = 2.35, p < 0.005$). **C**, lower doses (1–100 nM) of Rp-cAMPS (a PKA inhibitor) decreased extracellular glutamate in the NAcc ($F_{(16,84)} = 2.55, p < 0.05$). **D**, Rp-cAMPS (5 nM) blocked the APICA-induced increase in extracellular glutamate. The second APICA (100 μ M) administration produced a significant increase in glutamate 1 h later in the same rats ($F_{(16,67)} = 4.10, p < 0.001$). *, $p < 0.05$, compared with the average of the last three baseline samples (A and C) or the average of the three samples before APDC or APICA administration.

PKA is known to enhance transmitter release (Greengard et al., 1993) and could contribute to the Ca^{2+} -independent regulation of glutamate transmission by mGluRs observed in some studies (Scanziani et al., 1995).

Dimerization of Group II mGluR Immunoreactive Proteins Detected in Rat Brain. Previous anatomical studies have shown the existence of group II mGluR mRNA in the NAcc (Ohishi et al., 1993a, 1993b; Testa et al., 1998). The present study showed that there is a high density of mGluR2/3 immunoreactive proteins in the NAcc. The majority of mGluR2/3 in the NAcc, as well as in the prefrontal cortex, dorsal striatum and the ventral tegmental area appeared as a dimer. However, it is not known whether the dimer is a homodimer of mGluR2 or mGluR3 or a heterodimer of mGluR2/3, nor is the functional consequence of dimerization understood. Reports of hetero- and homodimerization of a variety of metabotropic receptors have emerged, and the functional consequences of dimerization that have been elucidated are generally consistent with promoting metabotropic receptor trafficking and signaling. For example, the hetero-dimer of GABAB receptor subtypes promotes the trafficking of active GABAB receptors into the plasmalemmal membrane (Kuner et al., 1999), and the dimerization of

δ -opioid receptors stabilizes receptors in the membrane (Cvejic and Devi, 1997).

In addition to acting as autoreceptors on glutamatergic presynaptic terminals, group II mGluRs are also expressed by astrocytes (Wroblewska et al., 1998). Recent evidence reveals that glia cells can release glutamate in a Ca^{2+} -dependent fashion using presynaptic protein assemblies similar to neuronal synaptic transmission (Araque et al., 1998). Moreover, mGluR receptors can activate Ca^{2+} currents in astrocytes, although most studies attribute this action to group I mGluR stimulation (Bernstein et al., 1998). Given that Ca^{2+} -dependent release of glutamate can occur in both glia and neurons, the in vivo estimates of extracellular glutamate in the present report cannot distinguish effects of mGluR2/3 agonists on neurons versus glia. Similarly, cystine-glutamate exchangers are present in both glia and neurons. Although the lack of effect by TTX supports a primary action on glia (which lack TTX-sensitive sodium channels), the sensitivity of the mGluR effects on glutamate to N-type Ca^{2+} channel blockade supports a role for neurons because N-type channels are absent or in very low abundance in glia (Araque et al., 2000).

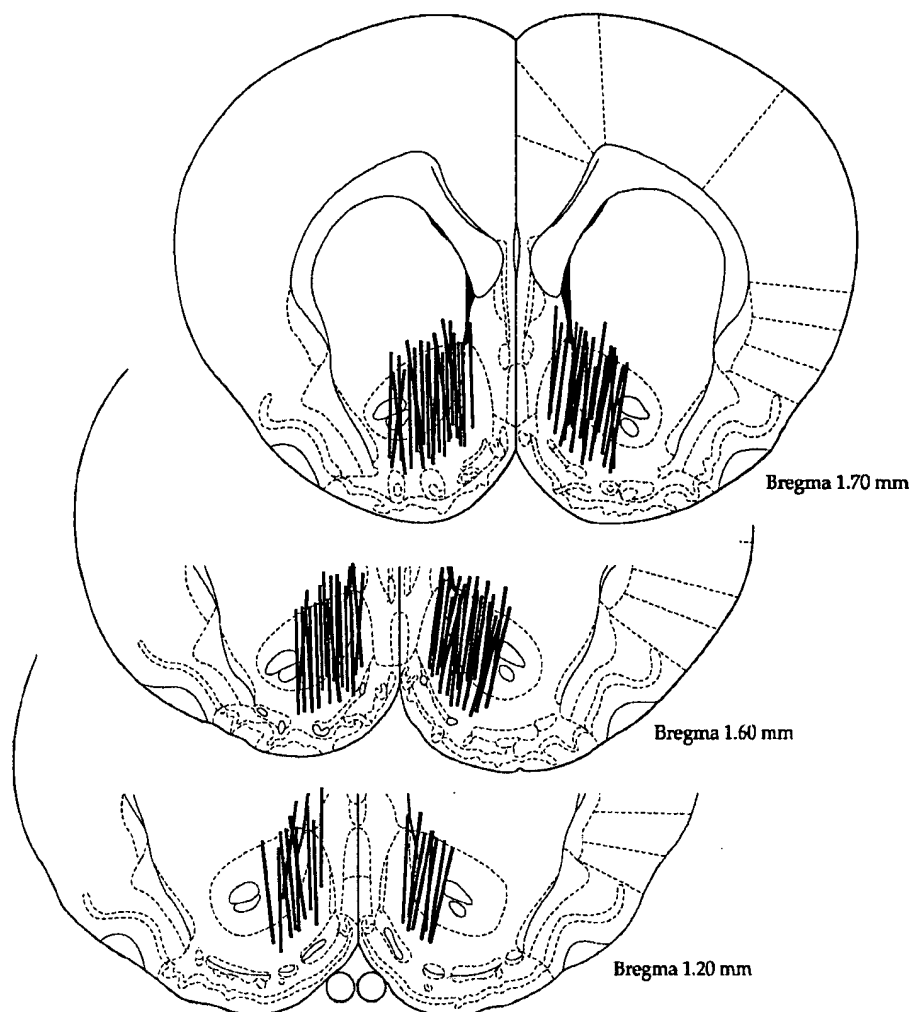


Fig. 8. Location of the microinjection cannula tips in the nucleus accumbens for microdialysis studies. The coronal drawings of the rat brain are based on the atlas of Paxinos and Watson (1986). The lines approximate the location of the 2 mm of active dialysis membrane in the nucleus accumbens.

Conclusions. Group II mGluRs were found to decrease both the Ca^{2+} -dependent vesicular release of glutamate and to involve the cystine-glutamate exchange, a main nonvesicular glutamate source. Moreover, the reduction in extracellular glutamate by stimulating mGluR2/3 was mediated by inhibiting PKA. Importantly, mGluR2/3 were found to bear significant in vivo glutamatergic tone because blocking mGluR2/3 elevated extracellular glutamate levels. This latter finding indicates that the extracellular pool of glutamate measured by microdialysis may regulate glutamate neurotransmission.

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Glutamate release inhibiting properties of the novel mGlu₅ receptor antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP): complementary in vitro and in vivo evidenceLisa S. Thomas ^a, David E. Jane ^b, Fabrizio Gasparini ^c, Martin J. Croucher ^{a,*}^a Department of Neuroinflammation, Imperial College School of Medicine, Charing Cross Hospital, Fulham Palace Road, London W6 8RF, UK^b Department of Pharmacology, School of Medical Sciences, Bristol BS8 1TD, UK^c Novartis Pharma AG, Therapeutic Area Nervous System, CH-4002 Basle, Switzerland

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Abstract

We have previously demonstrated that neuronal release of the excitatory amino acid glutamate is facilitated by the selective activation of presynaptic Group I metabotropic autoreceptors. Here we report the release *inhibiting* actions of the novel mGlu₅ receptor-selective antagonist, 2-methyl-6-(phenylethynyl)-pyridine (MPEP), both in vitro and in vivo. These data provide compelling evidence for the presence of functional positive modulatory mGlu₅ subtype autoreceptors in the mammalian central nervous system. © 2001 Published by Elsevier Science Ltd.

Keywords: Glutamate; Metabotropic glutamate receptors; Autoreceptors; Presynaptic; MPEP

Metabotropic glutamate (mGlu) receptors comprise a family of eight GTP-binding protein (G-protein)-linked receptors which are subdivided into three groups based on their amino acid sequence homology, signal transduction mechanisms and pharmacological properties (Conn and Pin, 1997). Group I receptor subtypes (mGlu₁, mGlu₅ and their splice variants) are selectively activated by (S)-3,5-dihydroxyphenylglycine ((S)-3,5-DHPG) and stimulate phospholipase C (PLC), increasing phosphoinositide hydrolysis and diacylglycerol (DAG) generation. Group II (mGlu₂ and mGlu₃) and Group III (mGlu₄ and mGlu₆₋₈) mGlu receptors are both negatively coupled to adenylate cyclase but can be distinguished pharmacologically by the use of selective agonists and antagonists (see Schoepp et al., 1999). Herrero and co-workers (1992) provided the first evidence for the existence of central mGlu autoreceptors which facilitate neuronal glutamate release with the demonstration that 4-aminopyridine-evoked glutamate release from rat cereb-

rocortical synaptosomes can be enhanced by (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid ((1S,3R)-ACPD), a non-selective mGlu receptor agonist. We have recently demonstrated that these presynaptic receptors are of the Group I-type in the rat forebrain slice preparation (Thomas et al., 2000) and in the rat corpus striatum in vivo (Patel and Croucher, 1998). These functional studies also suggested that the receptors mediating this response are unlikely to be of the mGlu₁ subtype as antagonists selective for these receptors were unable to influence the (S)-3,5-DHPG-evoked enhancement of electrically-stimulated [³H]D-aspartate ([³H]D-asp) release from rat forebrain slices. However, definitive evidence for the specific mGlu receptor subtype involved has been lacking due to the paucity of high-affinity selective ligands for the mGlu receptor subtypes. We now report the inhibitory actions of the recently described, potent and highly selective, non-competitive mGlu₅ receptor antagonist, 2-methyl-6-(phenylethynyl)-pyridine (MPEP) (Gasparini et al., 1999) against (S)-3,5-DHPG-induced release of [³H]D-asp in depolarised rat forebrain slices. The actions of this novel antagonist on glutamate release in vivo, as measured by intracerebral microdialysis, is also reported for the first time.

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A detailed account of the brain slice methodology utilised is given elsewhere (Patel and Croucher, 1997). Briefly, six coronal hemisections (500 μM thick) were obtained from the brains of male Wistar rats (240 ± 10 g; Charles River) and following a 45 min equilibration period in oxygenated brain modified Krebs bicarbonate buffer, these were “loaded” with [^3H]D-aspartate (Amersham International) by individual incubation at 37°C for 45 min in 5 ml of buffer containing 40 nM [^3H]D-aspartate (specific activity 25 Ci/mmol). Each hemisection was then transferred to a gold microelectrode superfusion chamber maintained at 37°C and superfused with oxygenated Krebs buffer at a rate of 0.4 ml/min for 45 min to wash off excess radiolabel. Superfusate samples were subsequently collected every 2 min. Basal superfusate samples were collected for 12 min before a 5 min period of electrical stimulation, comprising biphasic rectangular pulses (36 mA, 2 ms) at a frequency of 20 Hz, was delivered to the tissue. These parameters have previously been shown to provide a sub-maximal, highly Ca^{2+} -dependent, presynaptic GABA_B receptor-regulated, electrically-evoked release of [^3H]D-aspartate from rat forebrain slices (Patel and Croucher, 1997; Thomas et al., 2000). Samples were collected for a further 17 min before (S)-3,5-DHPG (0.1–3.0 μM) or MPEP (0.1–10 μM), alone or in combination, were added to the superfusate. Further basal samples were then collected over a 10 min period before a second period of electrical stimulation, with the same parameters as before, was applied to the tissue. Scintillation fluid was added to the collected superfusates and the radioactive content was quantified by liquid scintillation spectrometry. Mean basal efflux levels (B1 and B2) were calculated as the mean level of radioactivity in the 4 samples immediately preceding the respective periods of electrical stimulation. Responses to electrical stimulation (S1 and S2) were calculated as the total levels of stimulated efflux minus mean basal levels. Ratios of basal (B2/B1) and electrically-stimulated (S2/S1) efflux of label before and after drug application were calculated to assess the influence of the drugs on basal and stimulated release. Patel and co-workers (2001) have previously described in detail the construction and bilateral implantation of microdialysis probes into the striatum of rats for the procedure of *in vivo* microdialysis. In the following experiments, dialysis monoprobes (3 mm active length; Cuprophane membrane, 350 μM diameter; M.W. cut-off 40 kD) were inserted bilaterally via previously implanted guide cannulae to lie in the corpus striatum (tip position Fr, +1.5; Tr, ± 2.5 ; V, +6.0 relative to bregma) of male Wistar rats (275–300 g; Charles River). The technique for monitoring the efflux of isotopically labelled glutamate, preloaded in the brain *in vivo* by reverse dialysis, was first described and characterised by Young and co-workers (1990) and, with minor modifications, this methodology was adopted in the following study. All experiments were performed in

freely moving animals, over a 2 day period, 24 hours following probe implantation. Animals were connected for dialysis perfusion with artificial cerebrospinal fluid (aCSF) at a flow rate of 3 $\mu\text{l}/\text{min}$ for an initial 60 min equilibration period. The tissue was then preloaded with L-3,4-[^3H]glutamate ([^3H]L-glu; NEN Ltd) by perfusing with aCSF containing 5 μM [^3H]L-glu (specific activity 41.1 Ci/mmol) at a reduced flow rate of 1 $\mu\text{l}/\text{min}$ for 45 min. Dialysis perfusion was continued with normal aCSF for a further 45 min at 3 $\mu\text{l}/\text{min}$ to wash off excess radiolabel from around the perfusion site and then at 7.5 $\mu\text{l}/\text{min}$ for the remainder of the experiment. Following the washout period (time=0) samples were collected at consecutive 15 min time-points and scintillation fluid was added to 100 μl aliquots of each sample before quantification of the radioactivity content by liquid scintillation spectrometry. Using the current protocol, the majority of label collected in the dialysate samples, under both basal and stimulated conditions, is known to remain associated with L-glutamate (Young et al., 1990; Young and Bradford, 1993). To confirm the viability of the tissue being perfused, 100 mM potassium (K^+) was applied for 15 min by reverse dialysis through the monoprobes into the striatum at sample points 90 and 240 min. (RS)-3,5-Dihydroxyphenylglycine ((RS)-3,5-DHPG), at dialysate concentrations of 0.1–100 μM , was applied to the striatum at time point 165 min for 15 min, whilst MPEP, 10 μM , was applied at time point 150 min for a 30 min period. The radioactivity content of the samples after high- K^+ stimulation, agonist, or agonist/antagonist combinations was calculated as a maximum percentage change with respect to the preceding, baseline sample. When antagonist alone was tested, the average radioactivity content of the 2 samples over which it was applied to the tissue was taken and the percentage change in radiolabel release was calculated with respect to the preceding drug-free sample. All results are presented as the mean \pm SEM of n independent observations. Statistical significance of differences in responses was determined using analysis of variance (ANOVA) and a Student's post hoc *t*-test for independent groups. Values are considered to be significantly different from control if $P < 0.05$. All of the above experiments were performed in accordance with the regulations detailed in the Animals (Scientific Procedures) Act, 1986, and under appropriate project and personal licences.

Electrically-stimulated efflux of [^3H]D-aspartate from rat forebrain slices was enhanced in a concentration-dependent manner by the Group I mGlu receptor agonist (S)-3,5-DHPG (0.1–3.0 μM) with statistical significance being reached at 1 and 3 μM (Fig. 1A). Higher doses of the agonist did not evoke greater responses than those seen following (S)-3,5-DHPG, 3 μM (data not shown). The EC_{50} for (S)-3,5-DHPG for this response was 1.10 μM (GraphPad, Prism software). The agonist showed no significant effects on basal efflux of label within the dose

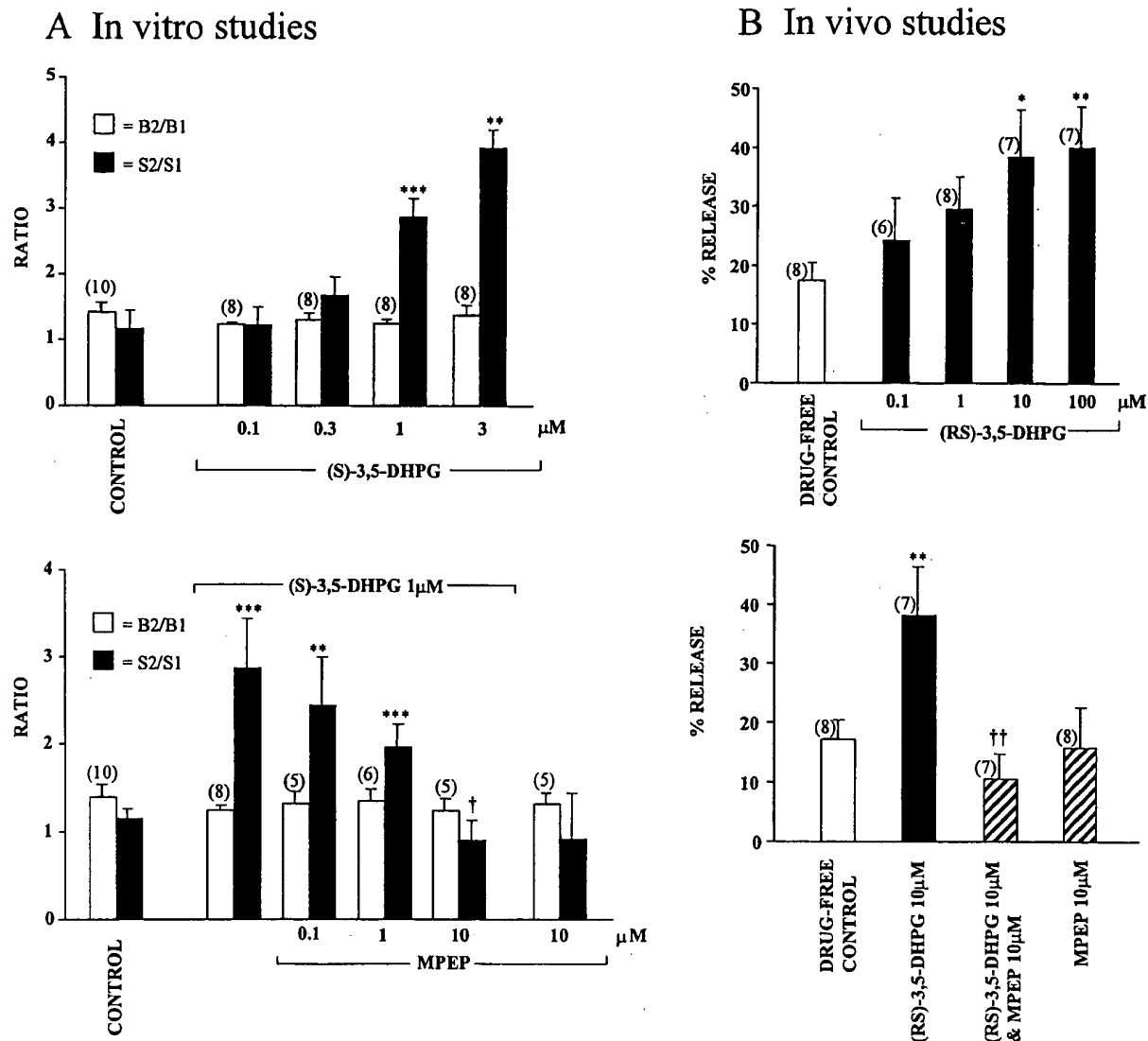


Fig. 1. Effects of the novel, selective mGlu₅ receptor antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP) on neuronal excitatory amino acid release in vitro (A) and in vivo (B). The first two panels (A) show the influence of the Group I mGlu receptor agonist (S)-3,5-DHPG on electrically-stimulated release of [³H]D-aspartate from rat forebrain slices and the concentration-dependent attenuation of this response by MPEP. In (B) the effects of (RS)-3,5-DHPG, administered by reverse dialysis, on the efflux of preloaded [³H]L-glutamate from the rat corpus striatum in vivo is shown, together with the powerful inhibitory action of MPEP on these (RS)-3,5-DHPG-evoked responses. Values shown are means (±SEM) of *n* (in parenthesis) independent observations. See text for details of procedures. * = *P* < 0.05, ** = *P* < 0.01, *** = *P* < 0.005 compared to control values; † = *P* < 0.05, †† = *P* < 0.01 compared to agonist alone (ANOVA followed by Student's *t*-test for independent groups). (S)-3,5-DHPG = (S)-3,5-dihydroxyphenylglycine; (RS)-3,5-DHPG = (RS)-3,5-dihydroxyphenylglycine; MPEP = 2-methyl-6-(phenylethynyl)-pyridine.

range tested (Fig. 1A). In order to test our hypothesis that presynaptic mGlu₅ receptors were responsible for the enhanced neurotransmitter release observed, the influence of the novel, selective mGlu₅ receptor antagonist MPEP (0.1–10 μM) against the responses evoked by (S)-3,5-DHPG, 1 μM was assessed. A maximal concentration of 10 μM was chosen due to the observed inhibitory effects of MPEP on NMDA receptors at higher concentrations, i.e. 100–200 μM (Gasparini et al., 1999; Attucci et al., 2001). The antagonist potently inhibited (S)-3,5-DHPG-enhanced release of electrically-stimu-

lated [³H]D-asp in a concentration-sensitive manner, with complete abolition of the response being achieved by MPEP, 10 μM (Fig. 1A). The calculated IC₅₀ value for MPEP for inhibition of (S)-3,5-DHPG-evoked responses was 0.24 μM (GraphPad, Prism software). The IC₅₀ value and the maximal inhibitory effect observed at 10 μM for MPEP confirm the exclusive involvement of the mGlu₅ receptors. Administration of MPEP alone, at the highest concentration utilised (10 μM), had no effect on the basal or electrically-stimulated release of radiolabel (Fig. 1A). To determine whether these novel

facilitatory mGlu₅ autoreceptors are functionally active *in vivo*, (RS)-3,5-DHPG (0.1–100 μ M) was applied to the corpus striatum of conscious, freely moving rats, via reverse dialysis through a microdialysis monoprobe. In a manner similar to that *in vitro*, (RS)-3,5-DHPG evoked a concentration-dependent increase in release of [³H]L-glu into the dialysis stream. When compared to drug-free control responses, significant increases in [³H]L-glu release were observed following (RS)-3,5-DHPG, 10–100 μ M (Fig. 1B). A maximal 21.0% increase in [³H]L-glu release compared to drug-free control response was attained following (RS)-3,5-DHPG, 100 μ M, whilst the estimated EC₅₀ concentration for (RS)-3,5-DHPG at this population of presynaptic facilitatory Group I mGlu receptors *in vivo* was calculated as 1.67 μ M (GraphPad, Prism software). Co-administration of MPEP, 10 μ M with (RS)-3,5-DHPG, 10 μ M resulted in full inhibition of the (RS)-3,5-DHPG-mediated increase in [³H]L-glu release (Fig. 1B). The antagonist, when applied alone at the same concentration, did not significantly influence [³H]L-glu release (Fig. 1B). No marked behavioural changes were observed following intrastriatal administration of (RS)-3,5-DHPG, MPEP, or the combined administration of these ligands by reverse dialysis, with all animals remaining alert but generally inactive throughout the experiments.

The data reported here provide compelling evidence for a role of presynaptic mGlu₅-type glutamate receptors in the regulation of neuronal glutamate release in the mammalian central nervous system. They also provide the first demonstration of functional presynaptic mGlu₅ receptors in conscious, freely moving animals. The absolute inhibitory actions of MPEP against the agonist-evoked responses in both the rat forebrain *in vitro* and during microdialysis of the corpus striatum *in vivo*, suggests that activation of presynaptic mGlu₅ receptors alone can fully account for the positive modulatory responses observed in these areas. Consistent with this conclusion, a recent study by Sistiaga and co-workers (1998) using knockout mice, clearly showed that nerve terminals from mGlu₁ receptor-deficient mice exhibit all the responses of Group I mGlu receptors observed in wild-type mice, including (RS)-3,5-DHPG-induced production of diacylglycerol (DAG) and the facilitatory influence of the agonist on neuronal glutamate release. Others have also shown that whereas presynaptic effects of (1S, 3R)-ACPD can still be observed in the hippocampal formation of mGlu₁ receptor-deficient mice (Aiba et al., 1994), these effects are absent in mGlu₅ receptor-deficient animals (Lu et al., 1997). In pharmacological studies, we have also previously demonstrated a lack of inhibitory activity of a wide range of mGlu₁ receptor-selective antagonists against presynaptic Group I mGlu agonist-evoked responses in the rat forebrain (Thomas et al., 2000). Additionally, in similar experiments, the selective mGlu₅ receptor agonist (RS)-2-chloro-5-hyd-

roxyphenylglycine ((RS)-CHPG) produced a marked enhancement of neuronal excitatory amino acid release (Thomas et al., 2000). The lack of activity of MPEP alone on drug-free control responses in the present studies indicates a lack of *tonic* activation of presynaptic mGlu₅ autoreceptors, either *in vitro* or *in vivo*, by the presumed endogenous agonist glutamate under resting conditions. Rather, presynaptic positive modulatory glutamate autoreceptors appear to permit the maintenance of “supply on demand” for neuronal glutamate release, so ensuring a maximal postsynaptic response to particularly intense presynaptic fibre activity. At least 2 different splice variants of mGlu₅ receptors (mGlu_{5a} and mGlu_{5b}) have been identified to date (Conn and Pin, 1997). The development of antagonists acting selectively at presynaptic mGlu₅ glutamate autoreceptor subtypes may provide new classes of drugs that could have a significant impact on the future treatment of a range of neurodegenerative disorders, including epilepsy and ischaemic brain damage, which are known to be associated with abnormally elevated levels of extracellular glutamate in the brain.

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Group I metabotropic glutamate receptors modulate glutamate and γ -aminobutyric acid release in the periaqueductal grey of rats

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Abstract

In this study, we investigated the effects of group I metabotropic glutamate (mglu) receptor ligands on glutamate and γ -aminobutyric acid (GABA) extracellular concentrations at the periaqueductal grey level by using in vivo microdialysis. An agonist of group I mglu receptors, (*S*)-3,5-dihydroxyphenylglycine [(*S*)-3,5-DHPG, 1 and 2 mM], as well as a selective agonist of mglu₅ receptors, (*RS*)-2-chloro-5-hydroxyphenylglycine (CHPG, 2 and 4 mM), both increased dialysate glutamate and GABA concentrations. 7-(Hydroxyimino)cyclopropa-[*b*]-chromen-1 α -carboxylate ethyl ester (CPCCOEt, 1 mM), a selective mglu₁ receptor antagonist, and 2-methyl-6-(phenylethynyl)pyridine (MPEP, 0.5 mM), a selective mglu₅ receptor antagonist, perfused in combination with DHPG, antagonized the effect induced by DHPG on the extracellular glutamate and GABA concentrations. MPEP (0.5 mM), perfused in combination with CHPG, antagonized the increased glutamate and GABA extracellular levels induced by CHPG. MPEP (1 mM) decreased the extracellular concentrations of glutamate but did not modify the dialysate GABA concentrations. Moreover, as the intra-periaqueductal grey perfusion of (*RS*)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid [(*RS*)-CPP, 100 μ M], a selective *N*-methyl-D-aspartate (NMDA) glutamate receptor antagonist, did not change the extracellular concentrations of glutamate, this suggests that the MPEP-induced decrease in glutamate is not a consequence of NMDA receptor blockade. These data show that group I mglu receptors in the periaqueductal grey may modulate the release of glutamate and GABA in awake, freely moving rats. In particular, mglu₅, but not mglu₁, receptors seem to be functionally active on glutamate terminals.

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1. Introduction

Glutamate plays a critical role as excitatory neurotransmitter in the central nervous system, and its effects are mediated by activation of ionotropic and metabotropic receptors (Nakanishi et al., 1998). Metabotropic glutamate (mglu) receptors are a heterogeneous family of G-protein-coupled receptors linked to multiple second messengers and modulation of ion channel function in the nervous system (Conn and Pinn, 1997; Knöpfel et al., 1995). Their classi-

fication into three groups (groups I–III) was determined by similarities in coupling mechanisms, molecular structure, homology of sequence and pharmacology (Nakanishi, 1994). Group I receptors (mglu₁ and mglu₅, and their splice variants) are positively linked to phospholipase C and, therefore, their activation results in increased phosphoinositide turnover. Localization of mglu₁ and mglu₅ receptors on the postsynaptic terminal seems to be under the regulation of a small family of "Homer" proteins (Brakeman et al., 1997; Ciruela et al., 1999). These glutamate receptors not only modulate the function of glutamatergic neurons but also are able to change the activity of inhibitory neurons via excitatory glutamatergic inputs to these neurons. Biochemical studies in a number of preparations consistently indicate that activation of groups II and III mglu receptors leads to

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suppression of the release of excitatory and inhibitory amino acids, whereas group I enhances the release of glutamate and γ -aminobutyric acid (GABA) (Cartmell and Schoepp, 2000). A specific role for mglu_1 and mglu_5 receptors in nociceptive processing has long been demonstrated in the dorsal horn by pharmacological, immunohistochemical and in situ hybridisation (Berthele et al., 1999; Bond and Lodge, 1995; Dickenson et al., 1997; Fisher and Coderre, 1996; Jia et al., 1999). The control of spinal cord nociception is subject to supraspinal, neuronal centres, including the mid-brain periaqueductal grey, the medullary nucleus raphe magnus and the adjacent medullary reticular formation (Basbaum and Fields, 1984; Duggan and Griersmith, 1979; Liebeskind et al., 1973). Since 1969, when it was demonstrated that stimulation of the periaqueductal grey produced analgesia, many studies have shown that this effect is the result of complex processes mediated by the periaqueductal grey (Reynolds, 1969). Besides glutamate and opioids, several other neurotransmitters in the periaqueductal grey participate in the control of nociception (Behbehani and Fields, 1979; Millan et al., 1987). Among these, GABA and glycine seem to play a crucial role in the processing of pain within this area (Maione et al., 1999, 2000; Moreau and Fields, 1986). In the current study, we analysed the possible participation of mglu_1 and mglu_5 glutamate receptors in the control of glutamate and GABA release in periaqueductal grey matter. In particular, we considered it interesting to explore whether periaqueductal grey functionally counteracting neurotransmissions, like glutamate and GABA, would be modulated at the same time by group I mglu subtype receptors. In our opinion, a better understanding of these processes might provide further insight into the pathophysiology of pain syndromes and possibly in changes in the functioning of the endogenous antinociceptive pathway. Previous studies in fact assessed the in vivo modification of glutamate or GABA (although not in the periaqueductal grey) induced by either stimulation or blockade of group I mglu receptors in some areas of the central nervous system (Battaglia et al., 2001; Cartmell and Schoepp, 2000; Cozzi et al., 1997, 2002; Herrero et al., 1992; Pellegrini-Giampietro et al., 1999). Although these previous findings confirmed the difficulty of determining the source of basal glutamate or GABA, they consistently found that group I mglu receptor stimulation increased both glutamate and GABA release. In particular, the group I mglu receptor-mediated increase in activity of GABAergic inputs could ultimately result in decreased excitatory neurotransmission.

Therefore, as the presence of mglu receptors in the periaqueductal grey has been shown by autoradiographic, immunostaining and pharmacological studies, and no study to date has investigated their possible role in the modulation of glutamate and GABA release at that level (Azcue et al., 1997; Catania et al., 1994; Leyva et al., 1995; Maione et al., 1998a, 2000), we evaluated this possibility in awake, freely moving rats.

2. Materials and methods

2.1. Animals

Male Wistar rats (250–300 g) were housed three per cage under controlled illumination (12:12-h light/dark cycle; light on 06.00 h) and environmental conditions (ambient temperature, 20–22 °C; humidity, 55–60%) for at least 1 week before the experiments started. Rat chow and tap water were available ad libitum. The experimental procedures were approved by the Animal Ethics Committee of the Second University of Naples. Animal care was in compliance with Italian (D.L. 116/92) and EEC (O.J. of E.C. L358/1 18/12/86) regulations on the protection of laboratory animals. All efforts were made to minimise animal suffering and to reduce the number of animals used.

2.2. Microdialysis procedure

Brain microdialysis experiments were performed in awake and freely moving rats as described previously (Biggs et al., 1992). In brief, rats were anaesthetised with chloral hydrate (400 mg/kg, i.p.) and stereotactically implanted with concentric microdialysis probes, which were constructed, as previously described, into the ventrolateral periaqueductal grey using coordinates A: –7.5 mm from bregma, L: +0.5 mm and V: 7.7 mm below the dura (Hutson et al., 1985; Maione et al., 1998b; Paxinos and Watson, 1986). Following a postoperative recovery period of approximately 18 h, dialysis was commenced with an artificial cerebrospinal fluid (ACSF, composition in millimolar: KCl, 2.5; NaCl, 125; MgCl_2 , 1.18; CaCl_2 , 1.26). ACSF (pH 7.2) was perfused at a rate of 0.8 $\mu\text{l}/\text{min}$ using a Harvard Apparatus infusion pump (mod. 22), and following an initial 60 min equilibration period (two discarded samples), 12 consecutive 30-min dialysate samples were collected. Rats received selective mglu receptor agonists, alone or in combination with the corresponding antagonists, directly through the dialysis probe (30-min perfusion). On completion of each experiment, rats were anaesthetised with pentobarbitone sodium and their brains were perfused-fixed via the left cardiac ventricle with heparinised paraformaldehyde saline (4%). Brains were removed 120 min following fixation, and coronal sections were cut in order to verify probe placements. Dialysates were analysed for amino acid content using a high-performance liquid chromatography (HPLC) method. The system comprised two Gilson pumps (mod. 303), a C18 reverse-phase column, a Gilson refrigerated autoinjector (mod. 231) and a Gilson fluorimetric detector (mod. 121). Dialysates were precolumn derivatised with *o*-phthaldialdehyde (10 μl dialysate + 10 μl *o*-phthaldialdehyde), and amino acid conjugates were resolved using a gradient separation. The detection limit of GABA and glutamate in 10- μl samples was about 0.5–1 and 2–3 pmol, respectively. The mobile phase consisted of two compo-

nents: (A) 50 mM sodium dihydrogen orthophosphate, pH 5.5, with 20% methanol and (B) 100% methanol. Gradient composition was determined with an Apple microcomputer installed with Gilson gradient management software, and the mobile phase flow rate was maintained at 1.0 ml/min. Data were collected by a Dell Corporation PC system 310 interfaced to the detector via a Drew data collection unit.

2.3. Immunohistochemistry

Animals were anaesthetised with sodium pentobarbitone and perfused with 4% paraformaldehyde, 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were removed, and transverse sections (60 μ m thick) of mesencephalon were cut using a vibratome. After preincubation with normal goat serum, the sections were incubated overnight at 4 °C with either anti-mglu₁ or anti-mglu₅ polyclonal antibodies (Upstate Biotechnology, Lake Placid, NY). After two washes with phosphate buffer solution, the sections were incubated with a biotinylated secondary antibody by using the avidin–biotin peroxidase procedure

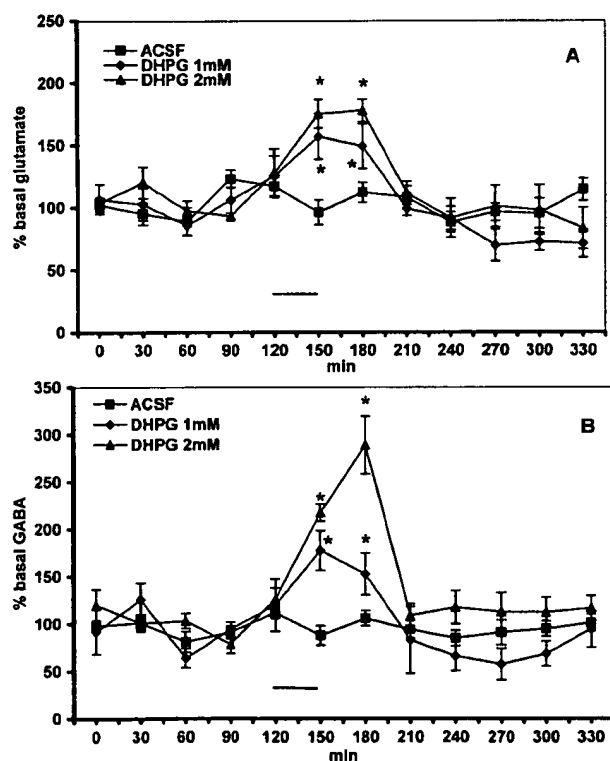


Fig. 1. Effect of artificial cerebrospinal fluid (ACSF) (A and B) or (*RS*)-3,5-DHPG (DHPG, 1 and 2 mM) on periaqueductal grey matter dialysate levels of glutamate (A) and GABA (B). The bars indicate the period of (*RS*)-3,5-DHPG perfusion. Data (five to eight rats per group) are means \pm S.E.M. of amino acid extracellular concentrations expressed as percentages of the basal values. The mean basal extracellular GABA and glutamate levels (not corrected for probe recovery) were 6.1 ± 0.7 and 32 ± 5 pmol/10 μ l of dialysate (means \pm S.E.M.), respectively. *Significant difference versus the vehicle. *P* values < 0.05 were considered statistically significant.

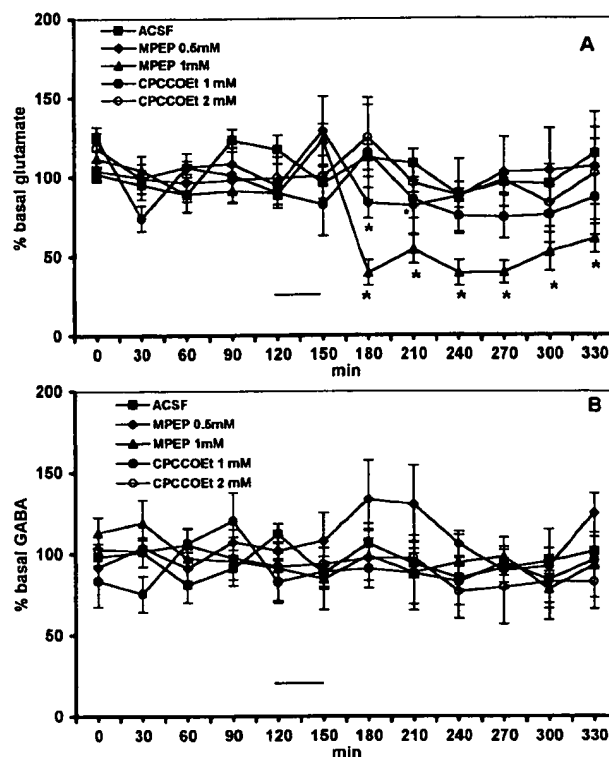


Fig. 2. Effect of artificial cerebrospinal fluid (ACSF) (A and B) CPCCOEt (1 and 2 mM) or MPEP (0.5 and 1 mM) on periaqueductal grey matter dialysate levels of glutamate (A) and GABA (B). The bars indicate the period of MPEP or CPCCOEt perfusion. Data (six to eight rats per group) are means \pm S.E.M. of amino acid extracellular concentrations expressed as percentages of the basal values. The mean basal extracellular GABA and glutamate levels (not corrected for probe recovery) were 5.9 ± 0.7 and 33 ± 6 pmol/10 μ l of dialysate (means \pm S.E.M.), respectively. *Significant difference versus the vehicle. *P* values < 0.05 were considered statistically significant.

(Vector Laboratories, Burlingame, CA) and diaminobenzidine as chromogen. The sections were then treated with 1% osmium, stained with uranyl acetate, dehydrated in graded solutions of acetone and embedded in araldite. Ultrathin sections were cut and analysed using a Philips CM10 electron microscope. The negative controls were made by omitting the primary antibodies and using nonimmune antisera, which resulted in complete absence of staining.

2.4. Drugs

The following drugs were used: (*S*)-3,5-dihydroxyphenylglycine [(*S*)-3,5-DHPG], (*RS*)-2-chloro-5-hydroxyphenylglycine (CHPG), 7-(hydroxyimino)cyclopropa-[*b*]-chromen-1 α -carboxylate ethyl ester (CPCCOEt), 2-methyl-6-(phenylethynyl)pyridine (MPEP), tetrodotoxin and (*RS*)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid [(*RS*)-CPP] (Tocris Cookson, Bristol, UK). CPCCOEt was dissolved in 10% dimethylsulphoxide in ACSF (pH 7.2). All the other drugs were dissolved in ACSF with final pH of 7.2.

2.5. Statistics

Statistical analysis of the data was performed by analysis of variance (ANOVA) followed by the Student–Newman–Keuls multiple comparisons test. Differences were considered significant at $P < 0.05$.

3. Results

The mean basal extracellular GABA, glutamate and glutamine levels in the periaqueductal grey (not corrected for probe recovery of $28 \pm 6\%$, $32 \pm 4\%$ and $30 \pm 7\%$ for GABA, glutamate and glutamine, respectively) were 6.4 ± 0.4 , 28 ± 5 and 422 ± 18 pmol/10 μ l of dialysate (means \pm S.E.M.), respectively. These values are in accordance with those obtained in our previous studies and in other laboratories (Renno et al., 1992; Maione et al., 1999, 2000). Each animal was used once only, and the reported basal

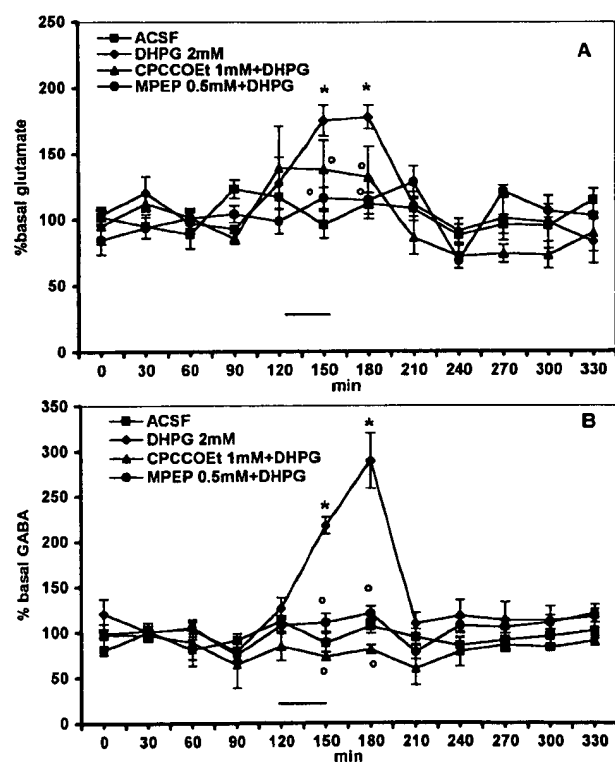


Fig. 3. Effects of artificial cerebrospinal fluid (ACSF) (A and B) (*RS*)-3,5-DHPG (DHPG, 2 mM), alone or in combination with CPCCOEt (1 mM) or MPEP (0.5 mM), on periaqueductal grey matter dialysate levels of glutamate (A) and GABA (B). The bars indicate the period of drug perfusion. Data (five to eight rats per group) are means \pm S.E.M. of amino acid extracellular concentrations expressed as percentages of the basal values. The mean basal extracellular GABA and glutamate levels (not corrected for probe recovery) were 6.9 ± 0.8 and 29 ± 6 pmol/10 μ l of dialysate (means \pm S.E.M.), respectively. *Significant difference versus the vehicle. *Significant difference versus DHPG 2 mM. P values < 0.05 were considered statistically significant.

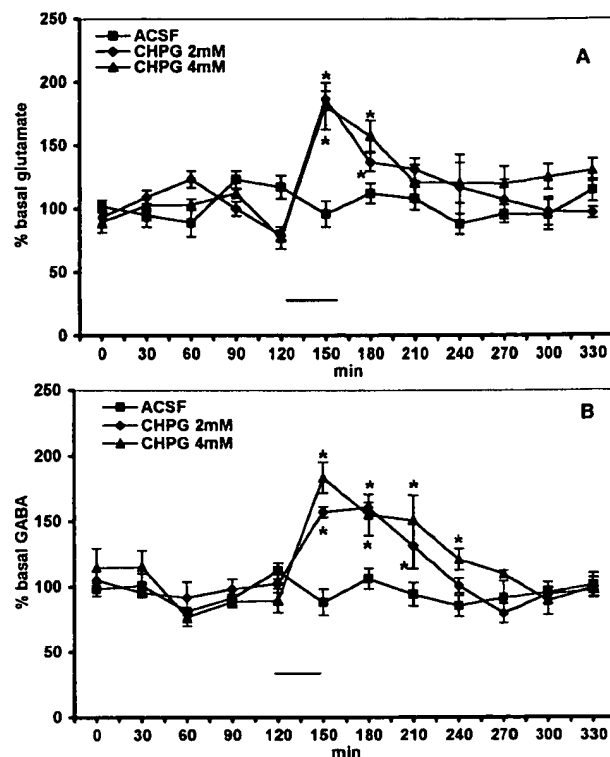


Fig. 4. Effect of artificial cerebrospinal fluid (ACSF) (A and B) or CHPG (2 and 4 mM) on periaqueductal grey matter dialysate levels of glutamate (A) and GABA (B). The bars indicate the period of CHPG perfusion. Data (five to eight rats per group) are means \pm S.E.M. of amino acid extracellular concentrations expressed as percentages of the basal values. The mean basal extracellular GABA and glutamate levels (not corrected for probe recovery) were 6.6 ± 0.8 and 26 ± 7 pmol/10 μ l of dialysate (means \pm S.E.M.), respectively. *Significant difference versus the vehicle. P values < 0.05 were considered statistically significant.

values of glutamate, GABA and glutamine are the mean concentrations obtained from all experiments. Intra-periaqueductal grey perfusion of tetrodotoxin (1 μ M) decreased the extracellular levels of glutamate and GABA ($48 \pm 6\%$ and $53 \pm 7\%$ for glutamate and GABA, respectively), but not glutamine (data not shown). Intra-periaqueductal grey perfusion of (*S*)-3,5-DHPG (1 and 2 mM), a selective agonist of group I mglu receptors, increased the dialysate glutamate ($156 \pm 18\%$ and $177 \pm 10\%$, respectively) and GABA ($177 \pm 23\%$ and $288 \pm 20\%$, respectively) concentrations (Fig. 1). CPCCOEt (2 and 4 mM), as well as its vehicle (dimethylsulphoxide, 10% in ACSF), did not modify per se dialysate glutamate and GABA levels (Fig. 2). MPEP (0.5–1 mM) decreased, in a concentration-dependent manner, the dialysate glutamate concentrations ($-58 \pm 7\%$) without affecting GABA concentrations (Fig. 2). Intra-periaqueductal grey perfusion of (*RS*)-CPP (100 μ M), a selective *N*-methyl-D-aspartate (NMDA) receptor antagonist, did not modify the extracellular levels of glutamate (data not shown). CPCCOEt (1 mM), a selective mglu₁ receptor antagonist, and MPEP (0.5 mM), a selective mglu₅ receptor antagonist, perfused in combination with (*S*)-3,5-

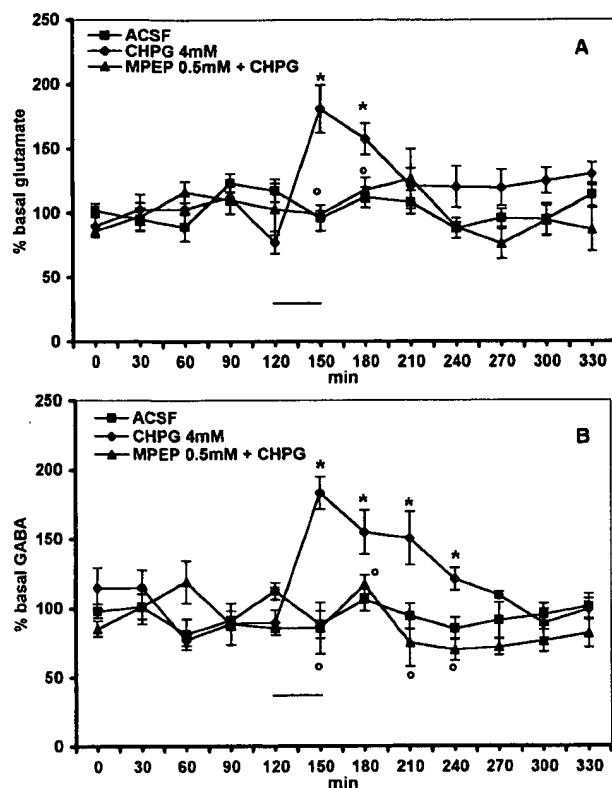


Fig. 5. Effects of artificial cerebrospinal fluid (ACSF) (A and B) or CHPG (4 mM), alone or in combination with MPEP (0.5 mM), on periaqueductal matter dialysate levels of glutamate (A) and GABA (B). The bars indicate the period of drug perfusion. Data (five to eight rats per group) are means \pm S.E.M. of amino acid extracellular concentrations expressed as percentages of the basal values. The mean basal extracellular GABA and glutamate levels (not corrected for probe recovery) were 6.9 ± 0.5 and 30 ± 40 pmol/10 μ l of dialysate (means \pm S.E.M.), respectively. *Significant difference versus the vehicle. °Significant difference versus CHPG 4 mM. *P* values < 0.05 were considered statistically significant.

DHPG, antagonized the (*S*)-3,5-DHPG-induced increase in extracellular concentrations of glutamate and GABA (Fig. 3). CHPG (2 and 4 mM), a selective agonist of mglu₅

receptors, increased the dialysate glutamate ($186 \pm 5\%$ and $180 \pm 18\%$, respectively) and GABA ($160 \pm 5\%$ and $183 \pm 11\%$, respectively) concentrations (Fig. 4). With (*S*)-3,5-DHPG or CHPG, the changes in amino acid extracellular values were greatest for GABA. MPEP (0.5 mM), a selective mglu₅ receptor antagonist, perfused in combination with CHPG (4 mM), antagonized the CHPG-induced increase in glutamate and GABA extracellular concentrations (Fig. 5). The extracellular concentrations of glutamine never changed following treatment with tetrodotoxin (1 μ M), (*S*)-3,5-DHPG, CHPG, CPCCOEt and MPEP (data not shown). At the ultrastructural level, immunostaining for mglu₁ and mglu₅ receptors was mainly localized in the dendrites of periaqueductal grey neurons (Fig. 6); however, some perikarya showed weak mglu₅ positivity. Many of the mglu₁- and mglu₅-positive dendrites received synapses containing round vesicles.

4. Discussion

There is an evidence that ionotropic and metabotropic glutamate receptors are expressed on both neural synaptic and astrocytic processes (Gallo and Ghiani, 2000). Although glutamate released from neurons can activate glutamate receptors on glia to cause changes such as (i) transmitter uptake into glial cells, (ii) modulation of K⁺ conductances and (iii) release of neuroactive substances from glia that can modulate synaptic transmission (Vernadakis, 1996; Araque et al., 1999), there is no way in this study to distinguish between glial or neural dialysate amino acids either before or after mglu receptor stimulation. Nevertheless, the changes in periaqueductal grey glutamate or GABA levels may deeply affect nociceptive perception, as this midbrain area is part of the endogenous antinociceptive system (Gebhart et al., 1984). In this study, the possible synaptic nature of periaqueductal grey dialysate glutamate and GABA seems confirmed in part

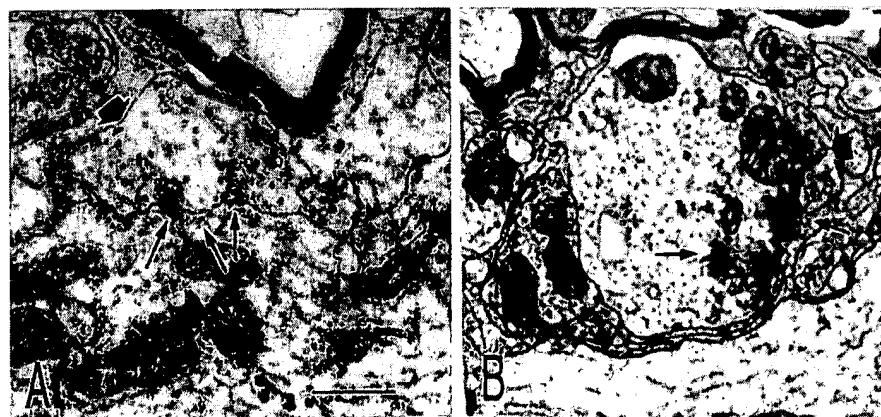


Fig. 6. Photomicrograph of mglu₁ (A)- and mglu₅ (B)-positive dendrites in the periaqueductal grey. The arrows indicate reaction product. The arrowhead indicates the axon terminal. Bar: 1 μ m.

by the fact that tetrodotoxin almost halved their extracellular concentrations. This finding suggests that almost 40% of extracellular GABA or glutamate in the periaqueductal grey may function as neurotransmitter. Moreover, this study provides pharmacological evidence in vivo that intra-periaqueductal grey perfusion of (*S*)-3,5-DHPG, an agonist of group I mglu receptors, and CHPG, a selective agonist of mglu₅ receptors, leads to an increase in glutamate and GABA extracellular concentrations. Contrary to what was observed with glutamate and GABA, the extracellular concentration of glutamine never changed with either (*S*)-3,5-DHPG or CHPG. This confirms that glutamine is not synaptically released and is mainly important in amino acid metabolic pathways (Maione et al., 2000). With (*S*)-3,5-DHPG or CHPG treatment, the change in amino acid extracellular levels was greatest for GABA. The postsynaptic nature of group I mglu receptors, as well as the huge population of GABAergic interneurons in the periaqueductal grey (possibly expressed at the somato-dendritic level), may underlie this difference (Lujan et al., 1996; Moreau and Fields, 1986). The selective mglu₁ receptor antagonist CPCCOEt was used in combination with (*S*)-3,5-DHPG in order to discriminate which mglu subtype receptor was involved. This antagonist was found to antagonize the effect of (*S*)-3,5-DHPG on both glutamate and GABA extracellular values. Since CHPG also increased extracellular concentrations of glutamate and GABA, the involvement of mglu₅ receptors was suggested. Compared to (*S*)-3,5-DHPG, CHPG induced a smaller increase in glutamate and GABA levels, and this may be the consequence of its lower potency (Doherty et al., 1997). Also, CHPG did not generate a concentration-dependent change in the extracellular levels of these amino acids, presumably because very similar concentrations (2 and 4 mM) of this drug were perfused. Intra-periaqueductal grey treatment with MPEP, a selective mglu₅ receptor antagonist, perfused in combination with CHPG, further confirmed the involvement of mglu₅ receptors in the modulation of glutamate and GABA release. Moreover, mglu₅ receptors seem to exert a tonic control of glutamate release at the periaqueductal grey level as their blockade with MPEP reduced per se the extracellular concentrations of this amino acid. The possible involvement of NMDA receptors in this effect was ruled out by the fact that (*RS*)-CPP, a selective NMDA receptor antagonist, did not decrease the glutamate extracellular level in the periaqueductal grey. The blockade of mglu₁ receptors with CPCCOEt had no effect either on glutamate or GABA extracellular level. In agreement with previous studies (Cozzi et al., 2002; Lorrain et al., 2002; Maione et al., 1998b; Neugebauer et al., 1999; Pintor et al., 2000), the concentration of mglu receptor ligands used in this study was higher than their in vitro EC₅₀ (millimolar versus micromolar) values. The fact that micromolar concentrations were ineffective may be possibly due to (i) the relatively low

probe recovery (20–30%), (ii) their uptake and metabolism by glial and neural cells or (iii) drug diffusion from probe site. We suppose that due to these reasons, the actual drug concentrations reaching cerebral tissue resemble those used in the in vitro studies (Schoepp et al., 1999). Since MPEP decreased extracellular concentrations of glutamate in the periaqueductal grey, a role of presynaptic mglu₅ receptors may be suggested in this response. In agreement with this concept, intracerebral microdialysis revealed a presynaptic mglu receptor-mediated enhancement of [³H]L-glutamate and endogenous transmitter release from the rat striatum and nucleus tractus solitarius (Jones et al., 1998a; Patel and Croucher, 1998). This is in agreement with a recent study by Thomas et al. (2000, 2001), showing that presynaptically located mglu₅ receptors positively modulate neuronal glutamate release in the rat forebrain in the presence of selective agonists. We recently showed that microinjections of MPEP into the periaqueductal grey of rat reduced the latency of the nociceptive reaction in the plantar test (Palazzo et al., 2001). This suggests that glutamate may physiologically modulate the endogenous antinociceptive pathway since decreased mglu₅ receptor activation may generate a persistent hyperalgesic effect. However, it also has been suggested that mglu₅ receptors serve as autoreceptors to inhibit synaptic transmission in the hippocampal area CA1 (Manzoni and Bockaert, 1995). Moreover, mglu₁ and mglu₅ receptors may exert opposite effects in primate spinothalamic tract neurons since (*S*)-3,5-DHPG potentiated and CHPG decreased the responses to brief innocuous and noxious mechanical stimulation (Neugebauer et al., 1999). In agreement with a previous neuroanatomical study (Azcue et al., 1997), we showed in this study that mglu₅, as well as mglu₁, immunoreactivity was located in cell bodies and dendritic processes. Axon terminals making synaptic contact with both mglu₁- and mglu₅-immunoreactive dendrites contained round clear vesicles. Therefore, in the light of these morphological findings, we can rule out the possibility that dialysate glutamate levels were modulated presynaptically by mglu₅ receptors.

In addition, this study suggests that group I mglu receptors regulate GABA release in the periaqueductal grey of awake, freely moving rat. This is shown by the finding that CPCCOEt and MPEP, selective antagonists of mglu₁ and mglu₅ receptors, respectively, antagonized the (*S*)-3,5-DHPG- and CHPG-induced increase in dialysate GABA levels. Previous studies showed either an augmentation of KCl-evoked GABA release or an increased release of this amino acid by selective mglu agonists in slices of rat striatum (Wang and Johnson, 1995; Wang et al., 1996). Also, 1*S*,3*R*-ACPD and the selective group I mglu receptor agonists (*S*)-3,5-DHPG and quisqualate increased KCl-evoked [¹⁴C]GABA release in superfused slices of rat nucleus tractus solitarius or potentiated increases in [¹⁴C]GABA release induced by NMDA in

striatal slices (Jones et al., 1998a,b; Hanania and Johnson, 1999). The role played by $mglu_1$ and $mglu_5$ receptors in the periaqueductal grey on the physiology of GABA release is far from established in awake, freely moving rat. There is evidence that microinjections of selective GABA receptor antagonists in the periaqueductal grey induce analgesia by decreasing the tonic GABAergic function (Moreau and Fields, 1986). In this study, we reported that group I $mglu$ receptors may positively modulate the release of GABA, which could be expected to generate hyperalgesia by inhibiting the antinociceptive pathway. However, this is in contrast with our previous observation that both acute and persistent nociceptive behaviours were decreased by intra-periaqueductal grey microinjection of group I $mglu$ receptor agonists (Maione et al., 1998a, 2000). It is possible that the variations in the extracellular levels of these amino acids may be a result of the complex network activity in the periaqueductal grey in vivo. Under our conditions, generalised stimulation of group I postsynaptic $mglu$ receptors in the periaqueductal grey may cause sustained activation of the output antinociceptive pathways. This may prevail over the other modulatory effects in the periaqueductal grey (i.e. the positive modulation of GABA release) and possibly mask the fine-tuning. The precise antinociceptive mechanisms induced by the selective $mglu_{1/5}$ agonists in the periaqueductal grey, in spite of the fact that GABA levels were increased, are unclear, and further investigation is needed to throw new light on the relationship between periaqueductal grey-induced analgesia and GABA and $mglu$ receptors. However, it is worth noting that $mglu$ receptor activation might either down-regulate or up-regulate inhibitory postsynaptic currents in the nucleus of the tractus solitarius and spinal cord in the rat (Glaum and Miller, 1993). Furthermore, activation of group I $mglu$ receptors was found to depress GABA_A-mediated inhibitory postsynaptic currents (IPSCs) in slices of rat midbrain dopaminergic neurons (Bonci et al., 1997). The fact that group I $mglu$ receptors modulate the release of excitatory and inhibitory neurotransmitters in the periaqueductal grey in the same direction may be suggestive of the physiological fine-tuning of these two counteracting neurotransmissions. As in other brain areas like the pallidum or substantia nigra (Hanson and Smith, 1999; Hubert et al., 2001), it is possible that group I $mglu$ receptors facilitate the release of GABA at extra synaptic sites or symmetric synapses in the periaqueductal grey. This $mglu$ receptor-mediated GABA release might be physiologically relevant to prevent excessive glutamate accumulation and, therefore, limit possible excitotoxic effects of high concentrations of glutamate.

In conclusion, this study provides evidence that $mglu_1$ and $mglu_5$ receptors may control the release of glutamate and GABA within the periaqueductal grey matter. In particular, $mglu_5$, but not $mglu_1$, receptors seem to be functionally active on postsynaptic terminals in this midbrain area, where

they tonically modulate the endogenous antinociceptive pathway.

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Differential Modulation of Ethanol-Induced Sedation and Hypnosis by Metabotropic Glutamate Receptor Antagonists in C57BL/6J Mice

Amanda C. Sharko and Clyde W. Hodge

Background: Emerging evidence implicates metabotropic glutamate receptor (mGluR) function in the neurobiological effects of ethanol. The recent development of subtype specific mGluR antagonists has made it possible to examine the roles of specific mGluRs in biochemical and behavioral responses to ethanol. The purpose of the present study was to determine if mGluRs modulate the acute sedative-hypnotic properties of ethanol in mice.

Methods: C57BL/6J mice were tested for locomotor activity (sedation) and duration of loss of the righting reflex (hypnosis) following acute systemic administration of ethanol alone or in combination with the mGluR5-selective antagonist, 2-methyl-6-(phenylethynyl)pyridine (MPEP), the mGluR1-selective antagonist, 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt), or the mGluR2/3-selective antagonist (2S)-2-Amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY341495).

Results: MPEP (10 and 30 mg/kg) significantly enhanced both the sedative and hypnotic effects of ethanol, while LY341495 (10 and 30 mg/kg) significantly reduced the sedative-hypnotic effects of ethanol. CPCCOEt had no effect at any concentration tested. Further loss of righting reflex experiments revealed that LY341495 (30 mg/kg) significantly reduced hypnosis induced by the gamma-aminobutyric acid type A (GABA_A) positive modulators, pentobarbital (50 mg/kg) and midazolam (60 mg/kg), and the N-methyl-D-aspartate (NMDA) receptor antagonist, ketamine (150 mg/kg), while MPEP (30 mg/kg) only significantly enhanced the hypnotic properties of ketamine (150 mg/kg).

Conclusions: These findings suggest that specific subtypes of the metabotropic glutamate receptor differentially modulate the sedative-hypnotic properties of ethanol through separate mechanisms of action, potentially involving GABA_A and NMDA receptors.

Key Words: Ethanol, Metabotropic Glutamate Receptors, mGluR5, MPEP, Sedation.

THE AMINO ACID glutamate is the primary excitatory neurotransmitter in the mammalian central nervous system. Glutamate receptors are divided into 2 categories: ionotropic receptors (iGluRs), glutamate-gated cation channels including NMDA, α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid, and kainate responsive receptors, and metabotropic receptors (mGluRs), a heterogeneous family of 7-transmembrane G-protein coupled receptors. Eight mGluR subtypes have been cloned. These receptors are divided into 3 broad groups based on amino acid sequence similarity, agonist pharmacology, and second messenger coupling. Group I mGlu receptors (mGluR1 and mGluR5) stimulate inositol phosphate metabolism and mobilization of intracellular Ca^{2+} , whereas group II (mGluR2 and mGluR3) and group

III (mGluR4, -R6, -R7, and -R8) inhibit adenylyl cyclase and reduce synaptic transmission (Gereau and Conn, 1995; Kew and Kemp, 2005; Pin and Duvoisin, 1995). In contrast to the iGluRs, which are responsible for the fast, excitatory responses to glutamate, mGluRs mediate the slower, modulatory responses to glutamate. In this capacity, mGluRs can modulate neurotransmission at both glutamatergic and nonglutamatergic synapses (Benquet et al., 2002; Diaz-Cabiale et al., 2002).

The availability of selective pharmacological agents has begun to reveal basic functional roles for the group I and II mGluR subtypes (Kew and Kemp, 2005). Group I selective agonists have been shown to increase glutamate and GABA levels in vivo (de Novellis et al., 2003) and enhance glutamate-evoked depolarization (Doherty et al., 1997; Pisani et al., 2001) and GABA-gated Cl^- currents (Hoffpauir and Gleason, 2002). Group II receptors are predominantly presynaptic autoreceptors (Cartmell and Schoepp, 2000) and selective activation of these receptors has been shown to reduce glutamate-evoked excitatory postsynaptic currents through inhibition of glutamate release (Shen and Johnson, 2003). mGluR selective ligands have also demonstrated behavioral activity in rodent models of anxiety and epilepsy. Group I antagonists and

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67

group II agonists have been shown to have anxiolytic (Klodzinska et al., 1999; Spooren et al., 2000) and anticonvulsant properties (Chapman et al., 2000; Moldrich et al., 2001).

Emerging evidence implicates mGluR function in ethanol's neurobiological and behavioral effects. Ethanol alters neuronal firing rates (Netzeband et al., 1997) and Ca^{2+} levels (Gruol et al., 1997) mediated by mGluRs in vitro. Chronic exposure to ethanol reduces mGluR1 mRNA levels in cerebellar Purkinje neurons of mice (Simonyi et al., 1996), and early withdrawal from ethanol leads to alterations in mGluR-evoked Ca^{2+} signaling in cerebellar neurons (Netzeband et al., 2002). In rats, chronic exposure to an ethanol-containing liquid diet decreased mRNA levels for mGluR3 and mGluR5 in the dentate gyrus, whereas mGluR1, mGluR5, and mGluR7 mRNA was decreased in the CA3 regions of the hippocampus (Simonyi et al., 2004). Neurobehaviorally, mGluR5 selective antagonists reduce ethanol self-administration in mice and rats (Cowen et al., 2005, 2007; Hodge et al., 2006; Lominac et al., 2006), decrease relapse to ethanol self-administration in rats (Backstrom et al., 2004; Schroeder et al., 2005), and block the discriminative stimulus effects of ethanol (Besheer and Hodge, 2005; Besheer et al., 2006).

The purpose of this study was to investigate the role of specific mGluRs in the acute sedative hypnotic effects of ethanol in mice. The 3 selective, systemically active mGluR antagonists 2-methyl-6-(phenylethynyl)pyridine (MPEP; mGluR5), 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt; mGluR1), and LY341495 (mGluR2/3) were examined for their ability to alter ethanol-induced inhibition of spontaneous locomotor activity (sedation) and ethanol-induced loss of righting reflex (LORR) (hypnosis). To explore potential mechanisms by which mGluRs might modulate responses to ethanol, these antagonists were also tested with other sedative-hypnotics: the GABA_A positive modulators pentobarbital and midazolam, and the NMDA antagonist ketamine. Blood-ethanol clearance studies were conducted to ensure that the observed effects were not due to alterations in ethanol clearance.

MATERIAL AND METHODS

Animals

Male adult C57BL/6J mice (Jackson Labs, Bar Harbor, ME; 10 to 24 weeks; 22 to 35 g) were housed 4 per cage and maintained on a 12-hour light/dark cycle with food and water available ad libitum. Animal care and handling procedures were performed in accordance with approved institutional protocols and the National Institutes of Health Guide for Care and Use of Laboratory Animals (1996).

Drugs

Ethanol (95% w/v) was diluted in physiological saline (0.9%) to a concentration of 20% (v/v) and administered in various volumes to obtain the appropriate doses. The mGluR1 selective antagonist CPCCOEt, mGluR5 selective antagonist MPEP, and the group II selective (mGluR2 and mGluR3) antagonist LY 341495 were purchased from Tocris (Ellisville, MO, USA). The mGluR5 selective antagonist 3-[(2-methyl-1,3-thiazol-4-yl)-ethynyl]-pyridine (MTEP)

was purchased from Ascent (Weston-super-Mare, UK). MPEP and MTEP were dissolved in physiological saline (0.9%). CPCCOEt and LY 341495 were suspended in (2-Hydroxypropyl)- β -cyclodextrin (45% w/v, Sigma, St Louis, MO) in distilled water. Pentobarbital, midazolam, and ketamine were purchased from Sigma-Aldrich and were dissolved in physiological saline (0.9%). Drug and vehicle solutions were administered to mice in a volume of 0.1 ml/10 g body weight, and dose selections were made based on pilot experiments and published studies (Naveilhan et al., 2001; Quinlan et al., 1998).

LORR Studies

Animals were treated with vehicle, MPEP (10 to 30 mg/kg), CPCCOEt (10 to 30 mg/kg), or LY341495 (10 to 30 mg/kg) 10 minutes prior to ethanol treatment (4.0 g/kg, IP) and the onset and duration of LORR (sleep time) were measured. Onset was calculated as the time between ethanol injection and LORR. LORR was defined as the inability of an animal to right itself within 30 seconds. Upon LORR, mice were placed in V-shaped troughs ($\sim 90^\circ$ angle) and the time to regain righting reflex was measured. Return of the righting reflex was defined as the ability of an animal to right itself 3 times in 30 seconds. Duration of LORR was calculated as the difference between loss and return of righting reflex.

The active antagonists (MPEP and LY341495) were then further tested with a series of ethanol doses (2.5 to 3.5 g/kg) to examine ethanol dose-dependence. MTEP (10 mg/kg) and MPEP (10 mg/kg) were also tested with ethanol (3 g/kg) to further confirm mGluR5 involvement. To examine possible mechanisms for the antagonist effects on ethanol-induced LORR, pentobarbital (50 mg/kg)-, midazolam (60 mg/kg)-, and ketamine (200 mg/kg)-induced LORR were also examined following pretreatment with the active mGluR antagonists.

Locomotor Activity Studies

Spontaneous locomotor activity was measured in 8 ENV 250 activity chambers (Med. Associates, St Albans, VT). Infrared light sources and photodetectors were used to measure horizontal distance traveled during test sessions. All sessions were 60 minutes. Sessions were recorded in 5-minute time bins at 100 ms resolution on a computer interfaced with the chambers. Following each session, chambers were cleaned with glacial acetic acid and rinsed with water.

All animals were chamber naive prior to testing. Vehicle, MPEP (30 mg/kg), CPCCOEt (30 mg/kg), or LY341495 (30 mg/kg) was administered by intraperitoneal injection (IP) 10 minutes prior to ethanol treatment (0 and 2.0 g/kg, IP). The animals were placed in the activity chambers immediately after ethanol injection.

Blood Ethanol Clearance

Vehicle, MPEP (30 mg/kg) or LY341495 (30 mg/kg) was administered by IP injection 10 minutes prior to ethanol treatment (4.0 g/kg). Blood ($\sim 30 \mu\text{l}$) was taken from the tail vein at various time points (10, 30, 60, 120, 180 minutes) after ethanol administration. Plasma from the samples was analyzed using an alcohol analyzer (Analox Instruments, Lunenburg, MA).

Analysis of Drug Effects

One-way ANOVA was used to determine the effects of the antagonists on single doses of sedative-hypnotic compounds. Two-way ANOVA was used to analyze the effects of the antagonists on total spontaneous locomotor activity and ethanol dose-dependent LORR. Three-way ANOVA with repeated measures was used to analyze the time course of antagonist effects on spontaneous locomotor activity. Two-way ANOVA with repeated measures and area under the curve (AUC) calculations were used to evaluate the blood-ethanol data.

Upon identification of statistical significance, post hoc comparisons were made with a Tukey's test where appropriate. In all cases, a value of $p < 0.05$ was considered significant.

RESULTS

Ethanol-Induced LORR

Systemic administration of the selective mGluR5 antagonist MPEP (0 or 30 mg/kg) or the mGluR2/3 antagonist LY 341495 (0 or 30 mg/kg) produced differential effects on the time required for animals to regain their righting reflex following a high dose of ethanol (Fig. 1). Pretreatment with the highest dose of MPEP (30 mg/kg) increased the duration of LORR induced by ethanol (4 g/kg) by 65% (Fig. 1A; $F[2,25] = 13$; $p < 0.001$). Follow-up analysis shows that 30 mg/kg MPEP was significantly different from saline and 10 mg/kg MPEP (Tukey; $p < 0.05$), indicating a dose-dependent effect of MPEP. In contrast, pretreatment with the mGluR2/3 antagonist, LY341495, decreased the duration of ethanol-induced LORR (Fig. 1B). LY341495 significantly reduced the duration of LORR produced by ethanol (4 g/kg) ($F[2,31] = 11$; $p < 0.001$), although responses to the 10 and 30 mg/kg doses (54.2 ± 5.0 minutes and 38.8 ± 5.3 minutes, respectively) were not significantly different from each other. Neither dose of the mGluR1 antagonist CPCCOEt tested (10 and 30 mg/kg) altered the duration of ethanol-induced LORR ($F[2,29] = 0.9$; $p = 0.415$) (Fig. 1C).

Ethanol-Induced Locomotor Inhibition

mGluR5 and mGluR2/3 antagonists differentially modulated the ability of a subhypnotic dose of ethanol (2.0 g/kg) to reduce spontaneous locomotor activity as measured by distance traveled in a novel environment. Two-way ANOVA revealed a main effect of ethanol on total ambulatory distance (Fig. 2A; $F[1,27] = 112$; $p < 0.001$). When administered prior to ethanol, MPEP (30 mg/kg) further reduced exploratory locomotor activity compared with ethanol control. Although the analysis shows no main effect of MPEP, there was a significant MPEP \times ethanol interaction ($F[1, 27] = 22$;

$p < 0.001$), indicating that the effect of MPEP on total motor activity depended on the dose of ethanol (Fig. 2A). Analysis of the time course of MPEP's effects on ethanol-induced sedation by 3-way RM ANOVA showed a significant interaction among MPEP, ethanol, and time (Fig. 2B, $F[11,297] = 2$; $p < 0.01$) and a main effect of time ($F[11,297] = 43$; $p < 0.001$), in addition to confirming the main effect of ethanol. Follow-up analysis of these data showed that MPEP pretreatment significantly enhanced ethanol-induced motor impairment during the first 5 minutes (Tukey; $p < 0.001$), as well as 25 and 55 minutes post injection (Tukey; $p < 0.05$). Pretreatment with LY341495 (30 mg/kg) reversed ethanol-induced locomotor inhibition, producing a main effect of LY341495 (Fig. 2C; $F[1,24] = 16$; $p < 0.001$) but no LY341495 \times ethanol interaction. However, time course analysis by 3-way RM ANOVA showed an interaction among LY341495, ethanol, and time (Fig. 2D; $F[11,220] = 2$; $p < 0.01$) and a main effect of time ($F[11,220] = 2$; $p < 0.001$), while also confirming the main effect of LY341495. LY341495 pretreatment significantly diminished ethanol-induced motor impairment at 5, 10, 15, 20, 25, and 35 minutes after ethanol treatment (Tukey; $p < 0.05$). Treatment with CPCCOEt (30 mg/kg) had no effect on total locomotor activity when administered alone or prior to ethanol treatment (Fig. 2E; $F[1,24] = 0.05$; $p = 0.83$) and temporal analysis showed no interaction among CPCCOEt, ethanol, and time (Fig. 2F).

Ethanol-Induced LORR: Ethanol Dose-Dependence

To further characterize the involvement of mGluR5 and mGluR2/3 receptors in ethanol-induced hypnosis, the highest effective dose of each antagonist was tested in combination with a range of ethanol doses. As shown in Fig. 3A, the duration of LORR was dose-dependently increased by ethanol ($F[3, 53] = 103$; $p < 0.001$). At a dose of 2.5 g/kg, ethanol did not induce LORR. Doses of 3.0, 3.5, and 4.0 g/kg of ethanol induced increasing durations of LORR. This dose-dependent effect of ethanol was enhanced by MPEP pretreatment. Two-way ANOVA showed a main effect of MPEP

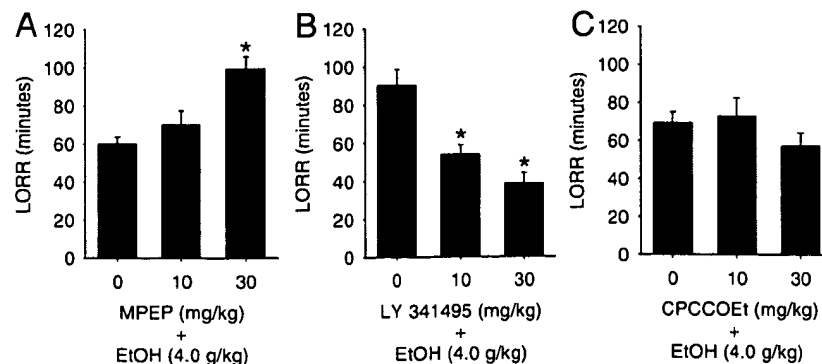


Fig. 1. Effects of mGluR antagonists on loss of righting reflex (LORR). Bars represent the mean (\pm SEM) duration of ethanol-induced LORR in minutes ($n = 6$ to 8) following pretreatment with MPEP (A), CPCCOEt (B), or LY341495 (C). *Significantly different from 4 g/kg ethanol alone ($p < 0.05$, Tukey's test).

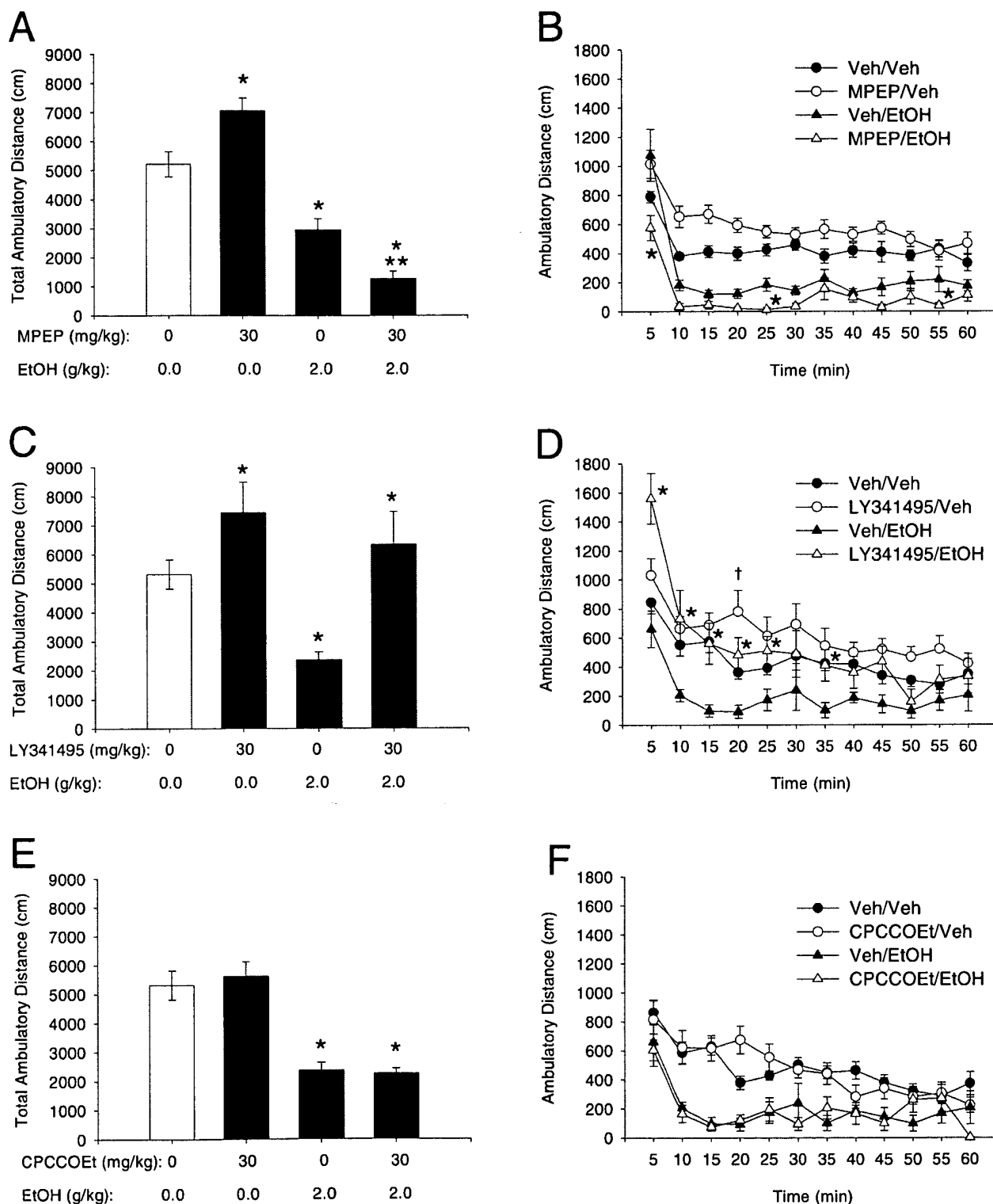


Fig. 2. Effects of mGluR antagonists on total locomotor activity alone and in the presence of a sedative dose of ethanol (**A**, **C**, and **E**). Bars represent the mean (\pm SEM) horizontal distance traveled in 60 minutes ($n = 6$ to 8) following pretreatment with vehicle, MPEP (30 mg/kg) (**A**), LY341495 (30 mg/kg) (**C**), or CPCCOEt (30 mg/kg) (**E**) with and without ethanol (2.0 g/kg). *Significantly different from vehicle/vehicle ($p < 0.05$, Tukey's test). **Significantly different from vehicle/ethanol ($p < 0.05$, Tukey's test). Temporal analysis of mean (\pm SEM) horizontal distance traveled in 5 minute time intervals ($n = 6$ to 8) following treatment with vehicle, MPEP (30 mg/kg) (**B**), LY341495 (30 mg/kg) (**D**), or CPCCOEt (30 mg/kg) (**F**) with and without ethanol (2.0 g/kg). *mGluR antagonist/ethanol treatment significantly different from vehicle/ethanol treatment at given time point ($p < 0.05$, Tukey's test). †mGluR antagonist/vehicle treatment significantly different from vehicle/vehicle at given time point ($p < 0.05$, Tukey's test).

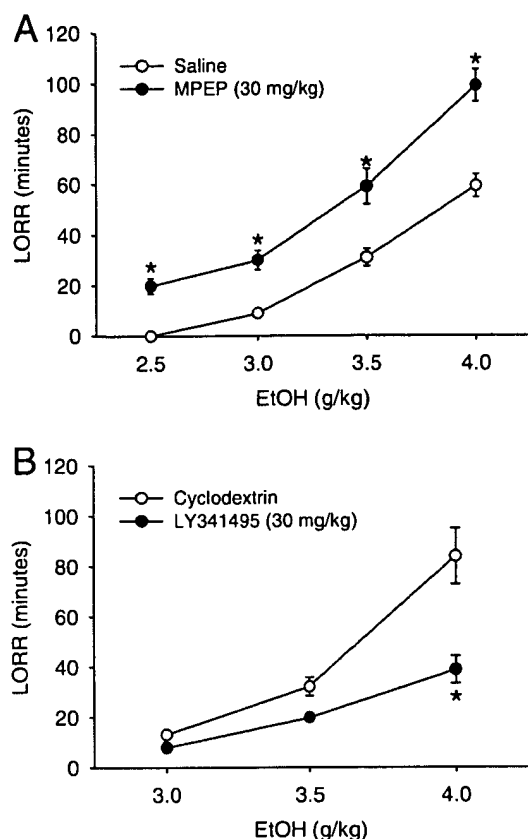


Fig. 3. Ethanol-induced loss of righting reflex (LORR) plotted as a function of ethanol dosage, symbols represent the mean (\pm SEM) duration of LORR in minutes ($n = 8$) following ethanol with saline pretreatment (open symbols) or in combination with MPEP (A) or LY341495 (B) pretreatment (closed symbols). *Significantly different from vehicle at corresponding dose of ethanol ($p < 0.05$, Tukey's test).

(30 mg/kg) ($F[1, 53] = 80$; $p < 0.001$). After MPEP pretreatment, the time to regain the righting reflex was significantly increased compared with the corresponding saline pretreated controls at all doses of ethanol tested (Tukey; p -values ≤ 0.002). MPEP pretreatment also altered the sedative-hypnotic effects of the lowest dose of ethanol, resulting in a LORR when combined with 2.5 g/kg ethanol. However, there was no MPEP \times ethanol interaction.

Figure 3B shows the ethanol dose-response curve following vehicle and LY341495 pretreatment. Two-way ANOVA showed that both ethanol and LY341495 produced main effects (EtOH: $F[2, 41] = 27$; $p < 0.001$); LY341495: ($F[1, 41] = 12$; $p = 0.001$). Analysis also showed a significant LY341495 \times ethanol interaction ($F[2, 41] = 5$; $p = 0.015$), indicating that LY341495 effects are dependent on ethanol dose. LY341495 (30 mg/kg) significantly reduced the duration of ethanol-induced LORR for a 4.0 g/kg ethanol dose (Tukey; $p < 0.001$), but not for the 3.0 or 3.5 g/kg doses.

Examination of the time to onset of LORR also demonstrated differences in the actions of MPEP and LY341495. As shown in Table 1, MPEP pretreatment resulted in a more rapid onset of LORR for the 3.0 and 3.5 g/kg doses of etha-

Table 1. Effects of mGluR Antagonists on Onset to Ethanol-Induced Loss of Righting Reflex

Ethanol (g/kg)	MPEP dose (mg/kg)		LY341495 dose (mg/kg)	
	0	30	0	30
3.0	2.4 \pm 0.1	1.9 \pm 0.1*	3.1 \pm 0.2	3.2 \pm 0.2
3.5	1.8 \pm 0.2	1.2 \pm 0.2*	2.6 \pm 0.2	2.5 \pm 0.2
4.0	1.5 \pm 0.1	1.2 \pm 0.1	1.9 \pm 0.1	2.0 \pm 0.2

Data expressed as mean \pm SEM minutes ($n = 8$ to 10).

*Significantly different from ethanol alone ($p < 0.001$, Tukey's test).

nol ($F[2, 39] = 20$; $p = 0.001$), while LY341495 pretreatment had no effect on onset time.

Ethanol-Induced LORR: mGluR5 Specificity

To further confirm the mGluR5 involvement, a low dose of MPEP and the highly potent and selective mGluR5 antagonist MTEP were tested in parallel with a lower hypnotic dose of ethanol (3 g/kg). As shown in Fig. 4, both MPEP (10 mg/kg) and MTEP (10 mg/kg) significantly increased the duration of LORR induced by ethanol (3 g/kg). The duration of ethanol-induced LORR was increased by 86% by MPEP and 133% by MTEP ($F[2,21] = 13$; $p < 0.001$). There was not a significant difference between the effects of MPEP and MTEP on ethanol-induced LORR suggesting that the effects observed following MPEP (30 mg/kg) are attributable to its actions on mGluR5.

Pentobarbital and Midazolam-Induced LORR

The GABA_AR positive modulator pentobarbital (50 mg/kg) induced an average duration of LORR comparable to that produced by a 4 g/kg dose of ethanol. Pretreatment with MPEP (30 mg/kg) did not alter the onset time or

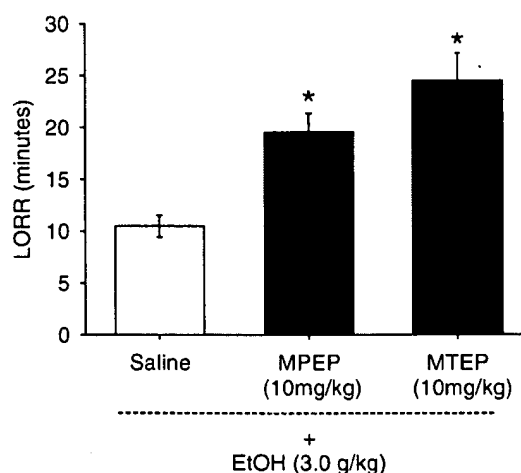


Fig. 4. Confirmation of mGluR5 specificity in loss of righting reflex (LOSS). Bars represent mean (\pm SEM) duration of ethanol-induced LORR in minutes ($n = 8$) following pretreatment with saline, MPEP, or MTEP. *Significantly different from saline pretreatment ($p < 0.05$, Tukey's test).

Table 2. Effects of mGluR Antagonists on Loss of Righting Reflex Induced by GABAergic and Glutamatergic Sedative-Hypnotics

	MPEP dose (mg/kg)				LY341495 dose (mg/kg)			
	0		30		0		30	
	Onset	Duration	Onset	Duration	Onset	Duration	Onset	Duration
Pentobarbital (50 mg/kg)	3.6 ± 0.4	63.9 ± 3.5	3.6 ± 0.5	56.3 ± 2.1	8.1 ± 0.7	46.2 ± 11.5	12.3 ± 0.9*	15.1 ± 7.6*
Midazolam (60 mg/kg)	10.6 ± 2.5	47.3 ± 3.2	6.2 ± 1.2	47.5 ± 3.6	25.5 ± 7.3	39.6 ± 8.2	0.0 ± 0.0*	0.0 ± 0.0*
Ketamine (200 mg/kg)	1.4 ± 0.1	41.0 ± 1.3	1.4 ± 0.1	56.8 ± 1.1*	2.7 ± 0.6	40.2 ± 1.9	2.1 ± 0.1	32.3 ± 1.3*

Data expressed as mean ± SEM minutes ($n = 8$ to 10).

*Significantly different from pentobarbital, midazolam, or ketamine alone ($p < 0.05$, Tukey's test).

duration of LORR in mice treated with pentobarbital (Table 2). In contrast, LY341495 (30 mg/kg) significantly increased onset time ($F[1,13] = 14$; $p = 0.002$) and shortened the duration of pentobarbital-induced LORR by 67% ($F[1,13] = 39$; $p < 0.001$). The GABA_AR benzodiazepine positive modulator midazolam (60 mg/kg) also produced an average duration of LORR similar to the highest dose of ethanol. Midazolam-induced LORR was unaffected by MPEP and completely reversed by LY 341495 (Table 2; Onset: $F[1,6] = 12$; $p = 0.013$; duration: $F[1,6] = 23$; $p = 0.003$).

Ketamine-Induced LORR

Like ethanol, the NMDA receptor antagonist ketamine (200 mg/kg)-induced hypnosis was differentially affected by pretreatment with MPEP and LY341495 (Table 2). MPEP (30 mg/kg) increased the duration of LORR in mice treated with ketamine by 39% ($F[1,13] = 85$; $p < 0.001$), while LY341495 (30 mg/kg) reduced ketamine-induced LORR by 20% ($F[1,13] = 12$; $p = 0.004$). Onset time was unaffected by either MPEP or LY341495 treatment.

Blood Ethanol Determination

To address the possibility that MPEP and LY341495 are eliciting their effects by altering ethanol metabolism, blood-ethanol concentrations were measured following a 4 g/kg dose of ethanol (Table 3). In vehicle treated animals, blood-ethanol concentrations decreased significantly over time (saline: $F[4,79] = 79$; $p < 0.001$; cyclodextrin: $F[4,88] = 42$; $p < 0.001$). Neither MPEP (30 mg/kg; $F[1,22] = 0.987$; $p = 0.331$) nor LY341495 (30 mg/kg; $F[1,14] = 1.3$;

$p = 0.274$) pretreatment altered the blood-ethanol clearance time course. However, comparisons of the blood ethanol concentrations of saline and cyclodextrin treated animals revealed significant differences between the 2 vehicle groups ($F[1,100] = 10$; $p = 0.003$; AUC: $F[1,22] = 11$; $p = 0.003$), specifically at the 120 and 180 minute time points ($p < 0.05$).

DISCUSSION

The purpose of this study was to investigate the role of specific mGluRs in the acute sedative-hypnotic effects of ethanol in mice by examining the effects of mGluR selective antagonists on ethanol-induced sedation and hypnosis. One of the primary findings of the present study is the mGluR5 antagonist MPEP increased ethanol-induced locomotor inhibition and LORR. These findings indicate that the inhibition of mGluR5 activity enhances the acute sedative-hypnotic effects of ethanol. Moreover, the more selective mGluR5 antagonist MTEP produced a comparable effect on ethanol-induced LORR, further supporting the conclusion that mGluR5 activity influences the sedative-hypnotic properties of ethanol. Although the exact role of mGluR5 in ethanol's pharmacological effects has not been identified, these results suggest that ethanol produces some of its acute inhibitory effects through negative modulation of mGluR5 activity. This hypothesis is supported by evidence showing that pharmacologically relevant concentrations of ethanol inhibit glutamate-induced Ca^{2+} -dependent Cl^- currents in *Xenopus* oocytes expressing mGluR5 (Minami et al., 1998). Furthermore, this inhibition of mGluR5 is PKC dependent. Because acute ethanol activates PKC γ in vivo (Wilkie et al., 2007), which desensitizes mGluR5 through phosphorylation (Gereau and

Table 3. Effects of mGluR Antagonists on Blood-Ethanol Concentration

	Time interval (min)					AUC
	10	30	60	120	180	
Saline	387.1 ± 18.6	381.6 ± 8.4	369.4 ± 6.9	314.7 ± 7.9	255.6 ± 8.8	57197.8 ± 1568.9
MPEP	391.2 ± 22.4	399.5 ± 14.6	387.1 ± 11.0	330.8 ± 11.3	275.5 ± 12.7	60347.3 ± 2627.5
Cyclodextrin	390.7 ± 10.9	406.2 ± 5.6	385.4 ± 8.4	353.0 ± 5.9*	314.6 ± 6.1*	62026.1 ± 680.4*
LY341495	399.9 ± 17.3	381.5 ± 11.6	379.3 ± 14.3	333.7 ± 16.0	305.8 ± 10.9	59802.8 ± 2008.8

AUC, mean area under the curve ± SEM.

Data expressed as mean ± SEM mg/dl ($n = 7$ to 16).

*Significantly different from saline (Tukey; $p < 0.05$).

Heinemann, 1998), it is possible that ethanol inhibits mGluR5 activity through PKC-dependent desensitization.

The other group I antagonist tested, the mGluR1 selective CPCCOEt, had no effect on ethanol-induced sedation or hypnosis. The absence of an effect for CPCCOEt is consistent with evidence suggesting that group I mGluR mediation of ethanol effects is specific to mGluR5. Acute ethanol treatment does not affect Cl^- currents in mGluR1-expressing oocytes (Minami et al., 1998), and our laboratory has shown no effect of CPCCOEt on ethanol consumption in either C57BL/6J mice or alcohol-preferring (P) rats (Hodge et al., 2006; Schroeder et al., 2005). However, our current results are inconsistent with a similar study (Lominac et al., 2006), which shows that pretreatment with CPCCOEt, but not MPEP, facilitates ethanol-induced motor impairment. While it is not entirely clear why these discrepancies exist, it may be due to differences in experimental procedure. We chose to use a higher dose of MPEP (30 mg/kg) based on our initial LORR study that showed no effect of a 10 mg/kg when paired with a 4 g/kg dose of ethanol. Furthermore, we employ a shorter pretreatment time. The onset to LORR and temporal distribution analysis of locomotor behavior both suggest that MPEP's behavioral effects are rapidly induced, implying that a shorter pretreatment time is necessary to see MPEP effects. Furthermore, our data showing that both a lower dose of MPEP and another mGluR5 selective antagonist, MTEP, increase the duration of LORR produced by a lower dose of ethanol (3 g/kg) support the conclusion that selective blockade of mGluR5 enhances ethanol's sedative-hypnotic effects. As for the differences in CPCCOEt effects, the longer pretreatment time and longer testing time used by Lominac et al. (2006) may be necessary for the expression of CPCCOEt enhancement of ethanol-induced motor impairment.

This study also shows that the mGluR2/3 antagonist, LY341495, decreased both the sedative and hypnotic effect of ethanol. Both doses of LY341495 (10 and 30 mg/kg) reduced ethanol-induced LORR. LY341495 (30 mg/kg) also reversed the sedative effects of ethanol, as measured by spontaneous locomotor activity. These results were not unexpected given that LY341495 has been shown to have locomotor activating properties (David and Abraini, 2001). A growing body of evidence indicates that group II mGluRs are presynaptic autoreceptors and blockade of these receptors with LY341495 increases glutamate release (Xi et al. 2002). Increases in presynaptic glutamate release are known to promote increased locomotor activity (Vezina and Kim, 1999), suggesting that reductions in ethanol-induced sedation and hypnosis by LY341495 are due to increased glutamatergic activity.

The ethanol dose-response data highlight other differences between the mGluR5 and mGluR2/3 effects. The mGluR5 antagonist MPEP produced a significant increase in the duration of LORR regardless of the dose of ethanol administered. Furthermore, the increases produced by MPEP were the same for each dose of ethanol. Although MPEP alone produced no sedative-hypnotic effects, combination with a sub-hypnotic dose of ethanol (2.5 g/kg) produced full hypnosis and a dura-

tion of LORR similar to the increases seen when MPEP was combined with fully hypnotic doses of ethanol. In contrast, the mGluR2/3 antagonist LY341495 produced a significant decrease in the duration of LORR only when administered with the highest dose of ethanol. These results indicate that the effects of LY341495 are dependent on the dose of ethanol administered. The onset to LORR data also present a distinction between the actions of MPEP and LY341495. Pretreatment with MPEP resulted in a more rapid onset of LORR for the 2 lower doses of ethanol (3.0 and 3.5 g/kg), providing further evidence that MPEP enhances the hypnotic properties of ethanol and does so rapidly. Pretreatment with LY341495 did not alter the onset of LORR at any of the ethanol doses tested.

Ethanol-induced hypnosis has been attributed largely to ethanol's ability to enhance inhibitory GABAergic responses and impair excitatory NMDA receptor activity (Beleslin et al., 1997). For an understanding of the mechanisms by which MPEP and LY341495 alter ethanol-induced hypnosis and a clarification as to whether these compounds selectively alter ethanol effects, MPEP and LY341495 were also tested with the GABA_A receptor positive modulators, pentobarbital and midazolam, and an NMDA receptor antagonist, ketamine, that exhibit hypnotic properties. MPEP pretreatment increased the duration of LORR for ketamine but had no effect on the hypnotic properties of pentobarbital or midazolam. This finding is consistent with reports that MPEP reduces NMDA-evoked responses (Attucci et al., 2001) and antagonism of mGluR5 and NMDA receptors have additive detrimental effects on learning and memory (Homayoun et al., 2004). Our results for pentobarbital and midazolam appear to be at odds with reports that mGluR5 activation positively modulates GABA_A receptor function in vitro (Hoffpauir and Gleason, 2002), although this may be specific to benzodiazepine-sensitive GABA_A receptors as MPEP inhibits the ethanol-like stimulus properties of diazepam, but not pentobarbital (Besheer and Hodge, 2005). However, it has also been reported that the anxiolytic effects of MPEP do not involve benzodiazepine-sensitive GABA_A receptors (Wieronska et al., 2004), indicating that further research must be done to determine the extent to which mGluR5 interacts with GABA_A receptors in vivo. Overall, these results suggest that blockade of mGluR5 increases the hypnotic properties of ethanol by enhancing ethanol-induced inhibition of NMDA receptors without affecting ethanol's actions at GABA_A receptors.

LY341495 reduced the duration of LORR for ketamine and pentobarbital and fully blocked induction of midazolam-induced LORR. These data are consistent with evidence showing that LY341495 has general stimulant effects (David and Abraini, 2001). While LY341495 reversed the hypnotic effects of all 3 drugs, it did so to varying degrees. LY341495 reduced ketamine-induced LORR by 20%, pentobarbital-induced LORR by 67%, and midazolam-induced LORR by 100%. Interestingly, LY341495 appears to be least effective at reversing hypnosis induced by NMDA receptor inhibition.

Based on our hypothesis that LY341495 reverses hypnosis by increasing glutamate release, these data suggest that the inhibition of mGluR2/3 results in enough glutamate release to counteract enhanced GABA_AR activity, but not enough to prevent decreased glutamatergic activity due to NMDAR inhibition.

One limitation of the present study that merits discussion is the potential off target effects of the pharmacological compounds tested. High concentrations of MPEP (20 μ M and above) have been associated with NMDA receptor inhibition (O'Leary et al., 2000) and positive allosteric modulation of mGlu4 receptors (Mathiesen et al., 2003). However, it has been reported that systemic administration of a 3 mg/kg dose of MPEP produces submicromolar concentrations in the brain (0.83 μ M; (Cosford et al., 2003), making it unlikely that a 30 mg/kg dose of MPEP would result in brain concentrations high enough to significantly affect NMDA or mGlu4 receptors. It has also been reported that MPEP can inhibit the norepinephrine transporter at nanomolar concentrations (Heidbreder et al., 2003), which may be related to the effects we report, given that the norepinephrine transporter has been implicated in the differential ethanol sensitivities of the long-sleep and short-sleep mice (Haughey et al., 2005). However, given that a low dose of MPEP (10 mg/kg) and the more selective mGluR5 antagonist MTEP (Varty et al., 2005) both enhanced the hypnotic effects of a low dose of ethanol, off target effects of MPEP do not appear to be a concern here.

Although there is currently no evidence that LY341495 has any off target effects, another mGluR2/3 antagonist has been shown to increase extracellular dopamine levels (Karasawa et al., 2006). Thus, additional research using gene knockout mice, RNA inhibition, or other more selective approaches are warranted to examine the selectivity of these compounds. The dose of ketamine used to induce LORR may also be having off target effects, namely at dopaminergic or nicotinic acetylcholine systems. As ketamine has been shown to increase extracellular dopamine release (Aalto et al., 2005), it is unlikely that interactions with dopaminergic systems are contributing to ketamine's sedative-hypnotic profile. Ketamine has also been shown to inhibit α 7-containing nicotinic acetylcholine receptors (nAChR) (Coates and Flood, 2001). This interaction may play a role in ketamine-induced hypnosis, as α 7-subunit null mutant mice are more sensitive to the sedative-hypnotic properties of ethanol (Bowers et al., 2005), and in the MPEP and LY341495 modulation of induced sedation, as recent evidence suggests a link between mGluR function and α 7-nAChR (Welsby et al., 2006). Again, further research is necessary to determine the roles of each of these systems.

Finally, neither MPEP nor LY341495 altered blood-ethanol clearance. However, cyclodextrin, the vehicle used for LY341495 and CPCCOEt, appears to slow the time course of ethanol elimination as compared with saline.

While these differences do not invalidate results from individual experiments, it limits the comparisons that can be made between saline vehicle and cyclodextrin vehicle experiments. These differences may contribute to the variability seen for the 3 vehicle groups in the locomotor activity experiments.

In summary, the present data suggest that specific subtypes of metabotropic glutamate receptors differentially modulate ethanol-induced sedation and hypnosis without altering the pharmacokinetics of ethanol elimination. Inhibition of mGluR5 enhances the sedative-hypnotic effects of ethanol, whereas inhibition of mGluR2/3 reverses these effects of ethanol. Our results also suggest that mGluR5 and mGluR2/3 elicit these changes through differential modulation of GABA_A and NMDA receptors.

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